UNIVERSITY OF EDINBURGH

AN INVESTIGATION OF THE MICROBIOLOGY OF BUILT-UP POULTRY LITTER

by

Henrietta Ethel Schefferle, B.Sc.

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INTRODUCTION

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The built-up litter system of poultry-keeping is one of the intensive methods of management whereby the birds are confined indoors during their productive life. In this way a high level of egg production can be maintained during the winter months.

The method consists in starting with about 4 to 6 inches of peat moss, chopped straw, wood shavings or some other form of litter and then adding to this periodically until a depth of 10 to 12 inches is attained. In this way excess moisture from the hens' droppings is absorbed and the litter is kept in a moderately dry and friable state. Under these conditions decomposition of the droppings takes place without the production of offensive odours.

The system originated in America, more or less by accident, when certain poultry houses were not cleaned out because of wartime labour shortage. It was noticed that the droppings did not accumulate and the birds on this litter continued to thrive. Since then the system has gained in popularity and is now used quite extensively in Great Britain. Its main advantages are that there is a saving of labour, farm buildings can be converted at low cost, removal of the litter may be reduced to once a year and heat is produced as a result of bacterial action. It has also been suggested that the litter can act as a source of nutritional factors.

The first part of the investigational work is concerned

with the numbers and types of micro-organisms found in poultry litter of varying nature and in poultry droppings. This is followed in Part II by a consideration of the decomposition of uric acid; as this compound is the main nitrogenous excretory product of birds its decomposition is probably largely responsible for the ammoniacal smell and high alkalinity of built-up litter. Part III deals with the characteristics and the possible identity of micro-organisms isolated from poultry litter and droppings.

Built-up poultry litter is reported to possess "animal protein activity". It is known that vitamin B12 is largely responsible for the special value of animal protein and for this reason the occurrence of this vitamin in built-up poultry litter is of interest. It was the object of Part IV to find out the extent to which vitamin B12 occurs in poultry litter and to study the rôle of micro-organisms in its synthesis.

PART I

TOTAL VIABLE COUNTS AND THE INCIDENCE OF VARIOUS GROUPS OF MICRO-ORGANISMS IN BUILT-UP POULTRY LITTER AND POULTRY DROPPINGS

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INTRODUCTION

The types of organisms found in frequently changed poultry litter will obviously depend on the types naturally present on the litter material and those added in poultry droppings. In addition, normal soil inhabitants would be expected to be present. In older or built-up litter this initial flora will become modified by the conditions in the litter. The temperature, the pH, the moisture content, which will also influence the degree of aeration, and the nature of the decomposable organic material present will all have some bearing on the microflora which becomes established.

The only investigation into the microflora of built-up poultry litter which has been found in the literature has been that of Halbrook, Winter & Sutton (1951) at Ohio. They examined litter made from ground corn cobs, shavings and also bark and found the same trend in the microflora in each case. In fresh litter under birds a general increase occurred up to 8 weeks in the total count at 37° and in the numbers of lactobacilli, enterococci, coliforms, moulds and yeasts. With built-up litter which had been in use for over a year the numbers of lactobacilli, coliforms, moulds and yeasts had decreased appreciably whilst that of enterococci only slightly. The aerobic plate count remained

virtually unchanged with increasing age of the litter and as the count was always much greater than could be accounted for by any of the groups determined, these workers postulated the existence of some other group of bacteria not shown up in their differential analysis. The general reduction in the counts of lactobacilli, coliforms, moulds and yeasts was accounted for by the increase in the pH of the built-up litter to an average of 8.0. The effect could be accentuated still further by raising the pH to values of about 8.6 by addition of lime. Unfortunately in this work the total aerobic count was determined at 37° at which temperature many organisms originating from soil fail to grow and the types comprising this count were not determined.

In the absence of any further information pertaining to the microflora of built-up poultry litter, it was thought that some knowledge of the normal flora of poultry droppings might be useful.

Rahner (1901) studied the flora of the droppings of chickens at various ages. No organisms were detected in the droppings of newly hatched chicks but after 2 days types of Bacterium coli appeared and after 4-5 days Gram-positive cocci and spore-forming rods were also present. Examining the faecal samples of 25 grown chicks he consistently isolated Bacterium coli gallinarum and showed it to be similar to Bacterium coli commune of man. Other organisms isolated were gelatin-liquefying cocci, Micrococcus candicans, Bacillus mesentericus, Bacillus megatherium, Bacterium fluorescens and once a yellow-pigmented gelatin-liquefying bacillus.

Occasional yeasts and moulds were detected but no anaerobes. Rahner considered that Bact. coli was the only intestinal organism and that all other types originated probably from the air and the food eaten.

King (1905) in studying the microflora of the intestinal mucosa of the chicken also found Bacterium coli communis to be the predominant inhabitant increasing in numbers from the duodenum to the caeca. He also found smaller numbers of spore-formers, micrococci and streptococci and occasionally pseudomonads, sarcinae and yeasts. The predominance of Bacterium coli in the faeces of chickens was also reported by Gage (1911).

Menes & Rochlin (1929) studied the intestinal microflora of chickens, geese and turkeys and found it to be the same from the duodenum to the rectum. The predominant inhabitants were Escherichia acidi lactici Hueppe and Streptococcus faecalis Andrewes and Horder. Occasionally were found colonies of Staphylococcus albus, Staphylococcus citreus, Sarcina flava, Bacillus subtilis, moulds, yeasts and actinomycetes. By first making an enrichment culture in acid broth they were also able to isolate Lactobacterium beijerincki (Bacillus beijerincki Henneberg) in 70% of cases.

Emmel (1930) found Escherichia coli and Escherichia communior to be the predominating organisms in the cloacal samples of 2 week-old chickens and hens. On an average 60% of the colonies developing on nutrient agar plates incubated at 37° were of these

two types. Aerobic spore-formers were frequently encountered but very few anaerobes were detected.

More recently Johansson, Sarles & Shapiro (1948) and Shapiro & Sarles (1949) have investigated the intestinal flora of chickens and hens. Using selective methods they estimated the numbers of lactic acid bacteria, enterococci, coliforms and yeasts and in addition determined the total aerobic count at 37°. Newly hatched chicks were found to have very few organisms in their intestinal tracts but the numbers of all groups of organisms increased very rapidly once food and water were given. By the use of improved culture media they were able to show that lactic acid bacteria comprised the largest group of intestinal micro-organisms in contrast to the findings of earlier workers. Coliforms and then enterococci were next in abundance. Yeasts were very variable in number depending on the diet. For battery birds coliform bacteria appeared to be predominately of the Escherichia coli and intermediate types, only an occasional typical Aerobacter aerogenes colony being observed. However with birds kept on range 10-25% of the colonies on the coliform plates were of the A. aerogenes type due no doubt to the accessability of the birds to soil and plant material where these organisms would be abundant. Enterococci were all identified as Streptococcus faecalis. On the agar plates incubated aerobically colonies of sarcinae, micrococci, actinomycetes, bacilli, coliforms and other unidentified types were encountered but no information is given as to the proportions in

which these various types occurred. Aerobic and anaerobic spore counts were of the same order of magnitude at all levels of the gastro-intestinal tract suggesting that bacterial spores are purely transitory in nature and pass ungerminated through the intestine of the bird.

An examination of the bacterial types in the caecal faeces of turkeys has been carried out by Harrison & Hansen (1950a). Selective plating methods were employed and total aerobic counts were determined at 41-42°. This temperature was chosen to discourage the growth of soil organisms ingested by the birds during feeding but which do not proliferate in the intestine. Anaerobic lactobacilli (the Lactobacillus bifidus type) were found to be the most numerous organisms making up about 50% of the total cultivable flora. Facultative anaerobic lactobacilli were also present but in relatively smaller numbers. Anaerobic streptococci and Gram-positive non-spore-forming rods other than lactobacilli were the next in abundance. Some of the organisms of this latter group had the morphology characteristic of Corynebacterium and have been placed in that genus. Gram-negative rods and facultative anaerobic streptococci existed in smaller numbers. Escherichia coli types made up the majority of the coliforms, Aerobacter and Proteus were never encountered. Of 32 facultative anaerobic streptococci isolated, 20 were identified as Streptococcus faecalis, 10 as Streptococcus liquefaciens and 2 as Streptococcus equinus. Members of the genus Micrococcus were

found to be very variable in their occurrence. Micrococcus epidermidis and Micrococcus pyogenes var. albus were isolated most frequently, Micrococcus aurantiacus and Micrococcus flavus only occasionally. Aerobic spore-forming rods were only occasionally isolated and were not thought to be indigenous to the caeca of turkeys.

From the available information it can be concluded that there are few bacteria in the intestine of the chick at the time of hatching but that counts rise rapidly once food and water are consumed. Lactobacilli appear to be the most numerous group at all levels of the intestine of the hen. The majority of these grow poorly or not at all under aerobic conditions (Shapiro, Rhodes & Sarles, 1949; Harrison & Hansen, 1950a, b) and in view also of the fact that they generally prefer slightly acid conditions for optimum growth, they would not be expected to multiply under the conditions of aeration and pH existing in built-up litter. Halbrook et al. (1951) have shown this to be the case, the numbers of lactobacilli decreasing under built-up conditions. The occurrence of this group of organisms has therefore not been considered in the following investigation.

Enterococci also occur in appreciable numbers in the intestine of the hen, and as these can multiply under aerobic and alkaline conditions it would be interesting to investigate their occurrence in built-up litter.

On aerobic plates used for estimating the total count, coliforms have consistently been found as the predominant organisms, the type being influenced by the management of the hens. These would be expected to multiply in the intestine. Other types found on these plates have been micrococci, sarcinae, aerobic spore-formers and occasionally pseudomonads, moulds, yeasts and actinomycetes. Many of these latter types are considered to be purely transitory in nature having originated from the food, water and soil. This belief is supported by the fact that many are strict aerobes and would be unable to proliferate in the intestine of the bird. Bacterial spores certainly appear to pass unaltered through the intestinal tract. It may be, that the actual number of these organisms is higher than has been indicated because estimates of total aerobic counts have all been made, as far as can be ascertained, at 37° at which temperature many of the organisms commonly occurring in soil and water fail to grow.

The aim of this section of the work was to study the kinds and numbers of various types of organisms in poultry litter of varying nature. One sample of poultry droppings was also examined.

EXPERIMENTAL METHODS

Collection of samples

When sampling built-up litter the surface layer, containing fresh droppings, was first removed. A sample was then taken from the remaining depth of litter, the process being repeated at several points throughout the poultry house in order to obtain as representative a sample as possible. The composite sample was then thoroughly mixed and a portion taken for bacteriological examination and for pH and moisture determinations. Handling of the samples was done as aseptically as possible.

At the time of sampling temperature measurements were also made. The temperature was taken at a point mid-way down in the litter. This was not always at the same depth as the total depth of litter varied considerably over any one house. Several measurements were made at random points throughout the house and the average taken.

For the one sample of poultry droppings examined, approximately 100 g. of fresh faeces were collected from the surface of the litter. After mixing, a representative sample was taken for bacteriological examination and moisture content. In the collection of this sample there was very little contamination from litter material.

Moisture content and pH of samples

For the determination of the moisture content of a sample, 50 g. of the material were held at 100^{o} overnight and the loss

of weight measured.

The pH measurements were made on a portion of the suspension (1/10 dilution) prepared for bacteriological examination, using a glass electrode.

Methods used for making plate counts

Samples were usually examined on the day following collection. Litter containing long lengths of straw was first cut into small pieces to facilitate the making of dilutions. A 30 g. sample was transferred to a previously sterilized glass macerator jar, 270 ml. of sterile water added and the whole mixed in a top-drive macerator for one minute. The succeeding dilutions were made by adding 10 ml. of the previous dilution to 90 ml. of sterile water in a glass stoppered bottle. Each dilution was mixed by vigorous shaking for one minute before preparation of the next dilution.

One ml. quantities of the appropriate dilutions, as determined by preliminary trials, were used for making pour plates in the customary manner.

In later work surface plating was used and in this case 0.1 ml. of the inoculum was spread over the surface of a dried prepared plate by means of a sterile glass rod bent at the end. This latter method had the advantage that surface colonies could be more easily recognized.

Platings of each dilution were usually made in triplicate and carried out as rapidly as possible after the dilutions were

prepared.

The conditions of incubation employed are given in conjunction with the experimental work, details of which follow.

Media used for making plate counts

The following media were used in making plate counts and will be referred to where applicable:

(1) Nutrient agar. Meat extract (Lab-Lemco), 10 g.; peptone, 10 g.; agar, 15 g.; water to 1000 ml.; pH 7.0.

(2) Peptone-yeast extract agar. Peptone, 2.5 g.; yeast extract (Difco Bacto), 2.5 g.; agar, 15 g.; water to 1000 ml.; pH 7.0.

(3) Glucose-yeast extract agar. Peptone, 10 g.; meat extract (Lab-Lemco), 10 g.; yeast autolysate, 50 ml.; glucose, 5 g.; agar, 15 g.; water to 1000 ml.; pH 6.0.

(4) Glucose-peptone acid agar. Glucose, 10 g.; peptone, 5 g.; K_2HPO_4, 1 g.; $MgSO_4.7H_2O$, 0.5 g.; agar, 15 g.; water to 1000 ml. The pH was adjusted to 3.8-4.0 by adding 1 ml. of 0.5N-H_2SO_4 to 100 ml. of the melted medium just prior to use.

EXPERIMENTAL AND RESULTS

Source and description of samples examined

The samples examined came mainly from Easter Howgate Farm but a few were obtained from other farms in the vicinity of Edinburgh. The source, description of sample and details of temperature, moisture content and pH are listed in Table 1. For built-up litter samples the date of starting the litter is also given.

Estimation of the total number of viable micro-organisms in poultry litter

Before determining the relative abundance of the different types of organisms occurring in high dilutions of poultry litter it was first of all necessary to select the most suitable medium and conditions of incubation for the development of the maximum number of organisms. With this object in view, colony counts of two different samples of litter (1c and 2) were made using the following media:

(a) Nutrient agar.

(b) Nutrient agar plus 2% urea autoclaved in the medium.

(c) Peptone-yeast extract agar.

In addition litter 1c was plated on three media containing an extract of litter. This extract was made by autoclaving a 1/10 suspension of built-up litter in tap water at 15 lb. for 15 min. and then filtering. The media containing litter extract had the following composition:

Table 1

Source and description of samples examined

Samples of poultry litter examined

No.	Source	Type of litter	Commenced	Age	Temp. °C.	Dry matter %	pH
1a	Easter Howgate, Midlothian	Chopped straw and peat moss	June 1953	Before Introdn. chicks	...	...	4.3
1b	"	"	"	3 months	...	...	8.4
1c	"	"	"	9 months	...	(very wet)	6.6
2	Easter Howgate, Midlothian	Not known	April 1953	2 months	...	...	8.6
3	Balerno, Midlothian	Not known	Jan. 1954	4 months	...	...	8.9
4a	Easter Howgate, Midlothian	Chopped straw and peat moss	May 1954	1 week	16.0	88.8	7.2
4b	"	"	"	5 weeks	19.6	79.0	7.9
4c	"	"	"	9 weeks	18.9	69.6	8.0
4d	"	"	"	3 months	20.0	72.8	7.6
4e	"	"	"	4½ months	12.9	76.8	7.6
4f	"	"	"	9 months	8.0	54.2	6.4

Table contd. on next page

Table 1 (contd.)

5	Easter Howgate, Midlothian	Chopped straw	August 1954	10 weeks	18.5	79.4	7.4
6	Traquair, Peebles-shire	Chaff, peat moss, and a little straw	November 1954	7 months	21.0	87.6	8.1
7	Dalkeith, Midlothian	Sawdust and cow manure	August 1954	9 months	26.0	58.2	8.5

Sample of poultry droppings examined

No.	Source	Dry matter %
8	Easter Howgate, Midlothian (Taken from the surface of litter no. 4a)	23.6

(d) Litter extract, 100 ml.; K_2HPO_4, 0.5 g.; agar, 15 g.; water to 1000 ml.; pH 7.0.

(e) As (d) plus 0.25% peptone.

(f) As (d) plus 0.25% yeast extract (Difco Bacto).

Plates were incubated at both 22° and 30° for 10 and 7 days respectively. No further development of colonies occurred after these intervals of time. Colony counts for the two samples of litter on the various media are given in Table 2.

On the basis of these results nutrient agar was chosen as the plating medium for the estimation of the total viable count. Peptone-yeast extract agar gave slightly lower counts while the addition of 2% urea to nutrient agar, designed to produce an alkaline reaction similar to that normally occurring in built-up litter, markedly reduced the counts. Colonies on this latter medium were also very small. Nothing was gained by the inclusion of litter extract in media and its use, therefore, was not continued.

Plates incubated at 30° gave a lower count than the corresponding plates at 22°. This was also found with other samples of litter showing that the higher temperature inhibited the growth of some organisms. An incubation temperature of 22° for 10 days was therefore adopted for all further work.

Total viable counts and the relative abundance of the bacterial types occurring on high dilution plates.

Total viable counts were made of thirteen samples of built-up litter, one sample of unused litter (1a), and one

Table 2

A comparison of the plate counts of two samples of litter on different media incubated at 22° and 30°

Counts expressed as millions/g. of fresh material

	Litter 1c		Litter 2	
	22°	30°	22°	30°
Nutrient agar	14,700	9,600	49,000	47,000
Nutrient agar plus 2% urea	5,000	2,100	40,000	20,000
Peptone-yeast extract agar	11,600	7,100	46,000	29,000
Litter extract agar	Pin-point cols.		...	...
Litter extract agar plus 0.25% peptone	13,000	6,000	...	...
Litter extract agar plus 0.25% yeast extract	13,900	10,000	...	...

sample of poultry droppings (8). The results are given in Table 3 as millions of colonies /g. of fresh material. Where moisture contents had been determined the results are also given in terms of the dry weight. The counts for built-up litter were all of the same order of magnitude ranging from 10,800 - 153,000 x 10^6 /g. of fresh material. The counts for unused litter and poultry droppings were both much lower being 188 x 10^6 and 88 x 10^6 /g. of fresh material respectively.

With the built-up litter samples there does not seem to be any marked effect on the total count by the nature of the litter; that is, the age, temperature, moisture content and pH. Litter samples 1c and 4f do show a slightly lower count and both had become slightly acidic and very wet. However as they were both sampled during the winter months when bacterial activity would be low on account of the temperature, it is not possible to draw any conclusions from these results as to the possible effect of pH and moisture content on the total count.

From the nutrient agar plates used for determining the total count, colonies were picked at random so that the relative abundance of the different bacterial types might be estimated. For the litter samples 1a, 1b and 1c colonies were also picked from plates of nutrient agar containing urea. Urea seemed to have no effect on the proportion of the different types and so the results for the two media have not been kept separate. Colonies were transferred to nutrient agar slopes

Table 3

Total viable counts of samples and the relative numbers of the predominant organisms

No.	Plate count x 10^{-6}/g. Fresh weight	Dry weight	Coryne-bacteria %	Micro-cocci %	Gram-neg. types %	Aerobic spore-formers %	Nocardia %	Strepto-myces %	Strepto-cocci %
*									
1a	188	...	16	8	68	0	0	8	0
1b	153,000	...	83	8	0	3	0	3	3
1c	14,700	...	56	2	40	2	0	0	0
2	49,000	...	72	3	12	3	5	0	5
3	21,000	...	66	31	0	0	0	0	3
4a	86,000	97,000	62	0	35	0	0	0	3
4b	35,000	44,000	41	3	48	0	0	3	3
4c	30,000	43,000	74	0	19	0	0	3	3
4d	36,000	49,500	65	0	35	0	0	0	0
4e	47,000	61,200	76	10	10	3	0	0	0
4f	10,800	19,900	60	0	31	0	6	3	0
5	72,000	91,000	76	7	14	0	0	3	0
6	35,000	40,000	74	17	9	0	0	0	0
7	36,000	62,000	80	8	4	0	8	0	0
8	88	372	72	9	19	0	0	0	0

* For description of samples see Table 1.

and incubated at 22°. As soon as growth appeared a Gram preparation was made for morphology and the young culture was examined for motility. Table 3 shows the number of the different types as percentages of the total number of colonies examined. Approximately thirty colonies from each sample were examined in this way.

The majority of organisms could be placed in one of three groups, corynebacteria, micrococci or Gram-negative types. Other bacteria such as aerobic spore-formers, nocardia, streptomyces and streptococci were found only occasionally. From the results it can be seen that corynebacteria are the predominant organisms in high dilutions of built-up litter and poultry droppings. The occurrence of micrococci is more erratic and there seems to be no relationship between their numbers and the properties of the litter. The percentages of the Gram-negative types vary widely and is perhaps influenced by pH. In many cases where the pH is high there are relatively fewer Gram-negative organisms. In unused litter there was a preponderance of Gram-negative types and relatively few corynebacteria.

Although the sample of poultry droppings showed a much lower total count than built-up litter samples, the proportions of the various types of organisms were very similar. Also, upon more detailed examination, as described in a later section of this work, strains isolated from poultry droppings appeared

to be identical with those normally found in built-up litter. This suggests that either the sample of poultry droppings had become contaminated from the litter material or that the organisms can be ingested from the litter and pass as transitory forms through the intestine of the bird. Settlement of this point would require the examination of the intestinal contents of hens kept on built-up litter. Time did not permit this to be done during the present investigation.

The count of Gram-negative organisms

From the examination of the colonies on the total count plates there was some indication that the relative abundance of Gram-negative organisms might be influenced by the pH of the sample. To gain more information regarding this it was decided to run parallel plate counts for Gram-negative organisms when total counts were being estimated. For this purpose crystal violet was added to media to give a final concentration of 1/500,000. The media tried were nutrient agar as previously described (p. 12) and the same medium with only half the concentration of peptone and meat extract. Plates were incubated for 10 days at 22°, the comparative results being shown in Table 4.

It was noticed that the dye became concentrated in many of the colonies and with crowded plates using ordinary nutrient agar this phenomenon must have reduced the effectiveness of the dye so that by 10 days' incubation Gram-positive organisms were

Table 4

Comparative counts for Gram-negative organisms on ordinary and half-strength nutrient agar containing 1/500,000 crystal violet

Sample no.	Plate count x 10^{-6}/g. fresh weight	
	Ordinary nutrient agar	Half-strength nutrient agar
4d	2,900	700
4e	870	670
4f	1,020	750

beginning to appear. However with plates showing only about a hundred colonies or less no difficulty of this kind was experienced and only Gram-negative organisms were able to grow. Streptococci were very occasionally found but their colonies were tiny and easily recognized. As the counts on ordinary nutrient agar were in all cases higher than the corresponding counts on the less concentrated medium, it was in future used for Gram-negative counts.

The results for ten samples of built-up litter and the one sample of poultry droppings are given in Table 5 in terms of fresh material and on a dry weight basis. The Gram-negative counts are also expressed as percentages of the corresponding total counts. This gives some idea of the efficiency of this plating method as this percentage can be compared with that for Gram-negative organisms obtained from examining colonies occurring on the total count plates (Table 3). When these two percentages are compared it can be seen that this plating method is not showing the expected number of Gram-negative organisms. The figures for the percentages of Gram-negative organisms resulted from an examination of only about thirty colonies on the total count plates and will thus be only very approximate. In view of this fact it is felt that the ratio of the two percentages is sufficiently constant for the assumption to be made that the plating method is giving a more or less constant proportion of the Gram-negative organisms. Thus although the figures do not represent absolute counts they can still be used for following

Table 5

Gram-negative counts of samples of built-up litter and poultry droppings and their relation to the corresponding total counts

Sample no.	Plate count x 10^{-6}/g. Fresh weight	Dry weight	Gram-neg. count as % of total count	Gram-neg. orgs. on total count plates %	Ratio of percentages
3	19.2	...	0.1	0	...
4a	8,900	10,000	10.3	35	0.30
4b	2,400	3,000	6.9	48	0.14
4c	1,000	1,400	3.3	19	0.17
4d	2,900	3,980	8.1	35	0.23
4e	870	1,130	1.9	10	0.19
4f	1,020	1,880	9.4	31	0.30
5	820	1,000	1.1	14	0.08
6	560	640	1.6	9	0.18
7	700	1,200	1.9	4	0.48
8	8.2	34.7	9.3	19	0.49

fluctuations in the number of Gram-negative organisms.

On examination of the results there seems to be no connection between the counts of Gram-negative organisms in built-up litter and the properties of the samples as given previously in Table 1. When the samples are listed in order of pH (Table 6) the Gram-negative counts and these counts expressed as percentages of the corresponding total counts fall in no particular order.

One interesting observation was made however in connection with another section of the work. In making an extract of litter for vitamin B12 assay, a suspension of 30 g. of litter in 270 ml. of water is adjusted to pH 5.0 by addition of N-H_2SO_4. If the figures for the volume of acid needed for this adjustment are used instead of pH values the results in Table 7 are obtained.

The relative abundance of Gram-negative organisms, as shown by the percentage figures, now definitely tends to decrease as the acid figures increase. It would appear therefore that the relative incidence of Gram-negative organisms in built-up litter is reduced by alkaline conditions and more effectively so if the material is well buffered.

From the plates used for estimating the Gram-negative counts, colonies (25-30 from each sample) were picked on to nutrient agar slopes and set aside for further study.

Organisms capable of growth at 45°

Enterococci have been reported to be present in considerable numbers in the intestinal contents of poultry and as this group

Table 6

Counts of Gram-negative organisms and the pH of samples

pH	Sample no.	Plate count x 10^6/g. Fresh weight	Dry weight	% of total count
6.4	4f	1,020	1,880	9.4
7.2	4a	8,900	10,000	10.3
7.4	5	820	1,000	1.1
7.6	4d	2,900	3,980	8.1
7.6	4e	870	1,130	1.9
7.9	4b	2,400	3,000	6.9
8.0	4c	1,000	1,400	3.3
8.1	6	560	640	1.6
8.5	7	700	1,200	1.9
8.9	3	19.2	...	0.1

Table 7

Counts of Gram-negative organisms and the volumes of acid required to adjust the pH of samples

Ml. N-H_2SO_4 added (pH 5)	Initial pH	Sample no.	Plate count x 10^{-6}/g. Fresh weight	Dry weight	% of total count
2.6	6.4	4f	1,020	1,880	9.4
8.8	7.6	4d	2,900	3,980	8.1
8.9	7.9	4b	2,400	3,000	6.9
9.1	7.2	4a	8,900	10,000	10.3
9.4	8.0	4c	1,000	1,400	3.3
11.8	7.6	4e	870	1,130	1.9
13.0	7.4	5	820	1,000	1.1
13.6	8.1	6	560	640	1.6
14.0	8.9	3	19.2	...	0.1
20.6	8.5	7	700	1,200	1.9

of streptococci can tolerate a high pH, they probably withstand the alkaline conditions in built-up litter. Plates of glucose-yeast extract agar, incubated for 2 days at 45°, were used for the detection of enterococci and certain other organisms capable of growth at this temperature. A pH of 6.0 was used. At neutrality coliform organisms (Escherichia coli, type I) tended to outgrow the streptococci but these were inhibited in the more acid medium. The results of counts on litter samples and poultry droppings and the types of organisms occurring on the plates are given in Table 8. Approximately ten colonies were examined from each sample.

Enterococci were the only organisms found on plates from poultry droppings and built-up litter samples. These were identified by their ability to grow at 45° and production of ammonia from arginine. All produced acid and reduced the dye in litmus milk, in most cases this was followed by curdling. Only one isolate showed proteolysis in litmus milk. Species differentiation within the group was not made. The plate counts given for built-up litter samples and poultry droppings can therefore be taken as estimates of the number of enterococci present. The extent to which they occur in built-up litter does not fluctuate widely and minor variations do not seem to be connected with any properties of the litter itself.

The count at 45° for unused litter was very much lower than that of built-up litter samples and did not, in this case,

Table 8

Plate counts and types of organisms capable of growth at 45°

Sample no.	Plate count x 10^{-3}/g. Fresh weight	Dry weight	Types of organisms present
Unused litter			
1a	15	...	B. licheniformis, E. coli type I, unidentified streptomyces
Built-up litter			
1b	163,000	...	Enterococci
2	423,000	...	Enterococci
4a	82,000	92,000	Enterococci
4b	13,000	16,000	Enterococci
4c	14,000	20,000	Enterococci
4d	7,000	9,600	Enterococci
4e	11,600	15,000	Enterococci
4f	2,700	5,000	Enterococci
5	60,000	76,000	Enterococci
7	56,000	96,000	Enterococci
Poultry droppings			
8	1,100	4,700	Enterococci

represent a count of enterococci. Organisms comprising this count were Bacillus licheniformis (identified by the key proposed by Gibson & Topping, 1938) Escherichia coli, type I and unidentified streptomyces.

Counts of moulds and yeasts

The numbers of moulds and yeasts were estimated by surface plating on a glucose-peptone medium acidified to pH 4.0. Plates were incubated for 5 days at 22° when yeasts and moulds could be easily distinguished by the nature of their colonies on the surface of the agar. Counts for moulds and yeasts, carried out on built-up litter samples and on poultry droppings, are given in Table 9.

Examining the figures for litter sample 4 there is a decrease in the numbers of moulds and yeasts with increasing age of the litter. This is most probably the result of the increase in alkalinity as the counts again rise in sample 4f where the pH had dropped to a value of 6.4. The other samples of built-up litter also show relatively low counts with high pH figures. Counts for poultry droppings are included for comparison.

Table 9

Mould and yeast counts for samples of built-up litter and poultry droppings

Sample no.	pH	Plate count x 10^{-3}/g. of dry material	
		Moulds	Yeasts
Built-up litter			
4a	7.2	12,400	99,000
4b	7.9	25,000	4,000
4c	8.0	7,800	1,400
4d	7.6	4,300	280
4e	7.6	1,200	117
4f	6.4	2,400	1,900
5	7.4	5,300	...
6	8.1	80	206
7	8.5	172	34
Poultry droppings			
8	...	550	4,450

DISCUSSION

Total bacterial counts of built-up poultry litter have been shown to be much higher than that of unused litter and to be fairly constant. The counts are also higher than those previously reported for built-up litter by Halbrook *et al*. (1951). This, however, is not unexpected as these workers incubated their plates at 37° at which temperature many organisms would not grow. Apart from slightly reduced counts which occurred for two samples taken during the winter months, any variation in total counts could not be related to the properties of the litter such as age, temperature or alkalinity. The organisms found on high dilution plates could be divided into three main groups, corynebacteria, micrococci and Gram-negative types. Although total counts showed little variation, the proportions of the different types of organisms on these plates altered with the alkalinity of the samples. Generally, the greater the alkalinity the higher was the proportion of corynebacteria and the lower the proportion of Gram-negative types. Although the incidence of Gram-negative organisms varied in this way, the exact relationship between their number and the pH and effective buffering action of their environment remained obscure.

The number of enterococci found in the sample of poultry droppings is of the same order of magnitude, $10^5 - 10^7$ /g. dry weight, as the numbers reported for the faeces of hens (Johansson *et al*., 1948) and the colon contents of chickens (Shapiro & Sarles,

1949). The numbers in built-up litter samples tend, on the whole, to be slightly higher and as the droppings would normally become diluted in the litter material it suggests that these types do continue to multiply after excretion. The counts for enterococci in built-up litter show no correlation with the nature of the litter samples. The results obtained during this investigation are in marked contrast to those reported by Halbrook et al. (1951) whose counts for enterococci for poultry droppings and litter samples were never greater than 100 /g. It is difficult to understand why the counts of Halbrook et al. (1951) were so much lower especially as their method of enumeration was the same as that used by Johansson et al. (1948) and Shapiro & Sarles (1949).

There was a tendency for the numbers of moulds and yeasts in built-up litter to be less at high pH values in agreement with the results of Halbrook et al. (1951). This was to be expected as the fungi are not favoured by very alkaline conditions and their numbers in soil tend to diminish with increasing pH (Waksman, 1931).

SUMMARY

Total viable counts have been determined for samples of unused and built-up poultry litter, and also for a sample of poultry droppings. For built-up litter, counts were of the order of 10^{10}-10^{11} /g. fresh weight and were little affected by the nature of the samples. The samples of unused litter and poultry droppings had lower counts.

In built-up litter of high alkalinity, corynebacteria were the predominant bacterial types. The occurrence of micrococci was found to be very erratic. The number of Gram-negative organisms appeared to be related to the alkalinity; they became less abundant in litters where the pH and buffering capacity were high.

Aerobic spore-formers, nocardia, streptomyces and streptococci were occasionally detected on high dilution plates used for estimating the total viable count of built-up litter samples.

The number of enterococci in built-up litter showed no great variation; minor fluctuations could not be related to any characteristics of the respective samples.

The numbers of moulds and yeasts tended to decrease in samples of built-up litter which had become strongly alkaline.

PART II

THE DECOMPOSITION OF URIC ACID BY MICRO-ORGANISMS FROM POULTRY LITTER AND DROPPINGS

THE DECOMPOSITION OF URIC ACID BY MICRO-ORGANISMS FROM POULTRY LITTER AND DROPPINGS

INTRODUCTION

The aerobic decomposition of uric acid

The early investigations into the aerobic decomposition of uric acid have been reviewed by Stapp (1920). It was found that aqueous uric acid solutions, if contaminated from the air, liquid manure or soil, underwent decomposition, the uric acid being replaced by first urea and then ammonium carbonate. In these early experiments pure cultures were not used and the breakdown to ammonia was most probably the result of the action of more than one organism. With the introduction of pure culture methods many organisms have been described as being capable of decomposing uric acid. The majority of these carry the decomposition only as far as urea but a few also produce urease with the result that ammonia is the nitrogenous end-product.

Little information exists about those organisms capable of producing ammonia from uric acid. Nawiasky (1908) stated that uric acid was decomposed by Bacillus proteus vulgaris with the formation of ammonia. By employing a temperature of 37° den Dooren de Jong (1929) was able to isolate an aerobic spore-former capable of utilizing uric acid which he named Bacillus fastidiosus. Uric acid was completely oxidized to carbon dioxide and ammonia.

Allantoin was not detected as an intermediate product but as the organism was able to utilize this compound it is conceivable that the decomposition proceeded first from uric acid to allantoin.

Stapp (1920) isolated six aerobic spore-formers from soil and faeces which could use uric acid as the sole source of carbon and nitrogen. Four of these have since been recognized as Bacillus megaterium types in Bergey's Manual (1948). The products of decomposition were not given.

The decomposition of uric acid by organisms belonging to the genus Pseudomonas has been reported by several workers. Liebert (1909), in studying the aerobic breakdown of uric acid to ammonia, found that the first stage resulting in the formation of allantoin and then urea could be brought about by Bacillus fluorescens liquefaciens, Bacillus fluorescens non-liquefaciens, Bacterium calcoaceticum and Bacillus pyocyaneus. By incorporating nitrate in the medium he was also able to demonstrate the anaerobic decomposition of uric acid by Bacillus pyocyaneus and Bacillus stutzeri, the nitrate simultaneously being reduced to molecular nitrogen. Den Dooren de Jong (1929) also verified Liebert's finding that pseudomonads are the most prominent types obtained in enrichment experiments carried out under aerobic conditions at low temperatures.

Stapp (1920) and den Dooren de Jong (1929) both mention the investigations of Ulpiani (1903) on uric acid decomposition.

He was probably the earliest worker to report a uric acid decomposer which from the description would appear to have been a corynebacterium or a nocardia. The organism, an aerobic bacterium isolated from chicken faeces, was described as being a non-sporing Gram-positive cocco-bacillus which tended to become coccal. It sometimes showed a filamentous form which later disintegrated into short thick rods. The organism formed yellowish-white colonies on uric acid agar and failed to liquefy gelatin. Uric acid was decomposed into urea and carbon dioxide; further hydrolysis of the urea did not take place. Den Dooren de Jong (1927) described an organism Mycobacterium opacum, later transferred to the genus Nocardia, which utilized uric acid as an energy source in a mineral salts medium. Clark (1955) has studied corynebacteria capable of decomposing uric acid; all were capable of growing on uric acid as a sole source of carbon and nitrogen and were urease-negative.

The majority of organisms decomposing uric acid aerobically form urea as an end-product. In a mixed population, as would exist in poultry litter, this compound would then be hydrolysed to carbon dioxide and ammonia by organisms having urease activity. This is a characteristic of many organisms belonging to a number of genera. Schneider & Gunderson (1946) found urease activity in cultures of Proteus spp., Aerobacter aerogenes and Brucella abortus whereas other faecal types were negative. Christensen (1946) also showed urease activity for cultures of Proteus spp.,

Aerobacter and "intermediate" types but not for cultures of Salmonella, Shigella and Escherichia.

Among the aerobic spore-formers Bacillus lentus, Bacillus sphaericus var. fusiformis, Bacillus pasteurii and some strains of Bacillus circulans are stated to be urease-positive (Bergey's Manual, 1948).

The ability to hydroyse urea is widespread among the parasitic corynebacteria (Jensen, 1952). Another coryneform organism, Bacterium ammoniagenes, which decomposes urea was described by Cooke & Keith (1927). Wood (1950) has also described corynebacteria associated with shark spoilage which produce urease.

Hydrolysis of urea appears also to be a common characteristic in the micrococcus-sarcina group of organisms (Shaw, Stitt & Cowan, 1951).

The anaerobic decomposition of uric acid

The anaerobic decomposition of uric acid was first studied by Liebert (1909). The breakdown was brought about by a spore-forming, motile, anaerobic rod which he named Bacillus acidi urici. The study was continued by Barker & Beck (1942) who isolated from soil a number of strains of anaerobic spore-formers capable of decomposing uric acid and some other purines. These organisms were placed in the new species Clostridium acidi-urici (Liebert) Barker & Beck and Clostridium cylindrosporum. The principal products of decomposition were ammonia, carbon dioxide

and acetic acid (Barker & Beck, 1941), xanthine being postulated by Beck (1950) as an intermediate product. The mechanism of the process is therefore very different from that involved in the breakdown of uric acid by certain aerobic organisms. These latter types carry out an oxidative decomposition involving the formation of first allantoin and then urea which may or may not be further decomposed to ammonia and carbon dioxide. Anaerobically oxygen is not used, urea is not formed and ammonia appears in appreciable amount.

Micrococcus lactilyticus, an anaerobic micrococcus, has also been reported to be capable of utilizing uric acid anaerobically (Whitely & Douglas, 1951) and Streptococcus allantoicus has been shown to decompose allantoin anaerobically (Barker, 1943).

EXPERIMENTAL AND RESULTS

In birds nitrogen is excreted mainly in the form of uric acid. As this would be the principal nitrogenous constituent in litter containing poultry droppings it is reasonable to assume that its subsequent decomposition is responsible for the ammoniacal smell and high alkalinity characteristic of built-up poultry litter. It was the object of this part of the investigation to study the organisms concerned in the breakdown of uric acid to ammonia in poultry litter.

The aerobic decomposition of uric acid

Organisms investigated

A large number of isolates were tested for their ability to decompose uric acid and also urea. The majority of these were isolated from high dilution plates used for estimating the total and Gram-negative counts. A few originated from uric acid agar plates and from enrichment cultures in a liquid uric acid medium. The relative numbers of the various types do not therefore represent the proportions in which they exist in poultry litter. Cultures were purified by at least two replatings before being examined in any detail.

Recognition of organisms capable of decomposing uric acid

The medium used for the recognition of aerobic uric acid decomposers was based on that used by Stapp(1920). This had the following composition: Uric acid, 1.0 g.; $Na_2HPO_4.12H_2O$,

6.0 g.; mineral solution, 100 ml.; distilled water to 1000 ml. The mineral solution contained KH_2PO_4, 1.0 g.; $CaCl_2$, 0.1 g.; $MgSO_4.7H_2O$, 0.3 g.; NaCl, 0.1 g.; $FeCl_3.6H_2O$, 0.01 g.; in 1000 ml. of distilled water. The mixture was boiled and adjusted to pH 7.0. To this was added 1.5% agar and the medium was sterilized at 22½ lb. momentarily.

To facilitate the recognition of uric acid decomposers plates were poured in two layers. Both layers consisted of this medium but the top layer contained also 1% uric acid in suspension added just prior to pouring. The addition of uric acid in this way did not affect the pH of the medium. This gave an opaque plate and any breakdown of uric acid resulted in a clearing of the medium which was easily detected. Cultures were streaked on plates of this medium and incubated for 7 days at 22°.

Later it was found that organisms, which when freshly isolated produced clearing on this medium, failed to do so after several transfers on artificial media. The addition of yeast extract produced good growth of these organisms accompanied by clearing and henceforth 0.25% Difco yeast extract was included among the constituents.

It was thought that a uric acid suspension in nutrient agar might be used for demonstrating uric acid decomposition. Here, however, difficulties were encountered. Some organisms which produced clearing on nutrient agar plus a suspension of

uric acid failed to do so when grown on the uric acid medium previously described. It was found that if the nutrient agar plates were flooded with N-H_2SO_4 recrystallization of uric acid occurred in the cleared areas. This did not occur when uric acid agar plates showing clearing were similarly treated. These cleared areas on nutrient agar were evidently due to the production of alkali which merely dissolved the uric acid in the vicinity of the bacterial growth. Treatment with acid caused reprecipitation of the insoluble uric acid.

A comparison was also made between the uric acid medium described and an agar containing the same mineral ingredients as the former but lacking the uric acid and Na_2HPO_4. Both media contained 0.25% Difco yeast extract and had a 1% suspension of uric acid in the upper layer. Although this latter medium was satisfactory for testing individual cultures fewer colonies showed clearing when used for a plate count of uric acid decomposers. The greater buffering capacity of the phosphate containing medium may have been responsible for this. The former medium was therefore adopted for future use.

(a) Organisms forming ammonia from uric acid

With some cultures a zone of precipitation occurred around the growth on uric acid agar plates (see Plate 1). This precipitate was much denser than the original opaqueness produced by the suspension of uric acid. On further incubation clearing usually occurred in the immediate vicinity of the

PLATE I

DECOMPOSITION OF URIC ACID BY MICRO-ORGANISMS

FORMATION OF AMMONIA

FORMATION OF UREA

bacterial growth and at the same time the dense precipitate spread outwards over the plate.

All cultures producing this effect were shown to possess urease activity. It therefore seemed probable that the breakdown proceeded through urea to ammonia which would combine with any residual uric acid to give the very insoluble ammonium urate. This interpretation was confirmed by a microscopic examination of the crystals together with the fact that ammonia was shown to be present by flooding the plates with Nessler's reagent. The ammonium urate crystals are burr-like in form or appear as spheres covered with spikes. Although urea was not actually demonstrated as a decomposition product by these organisms it seems most probable that the breakdown proceeds via this compound.

(b) Organisms forming urea from uric acid

Organisms which produced clearing on the opaque uric acid agar (Plate 1) were further tested for their ability to form urea as a degradation product. The organisms tested included 15 corynebacteria, 14 pseudomonads and 13 motile non-fluorescent Gram-negative rods. Included also in the experiment were a corynebacterium and 8 motile non-fluorescent Gram-negative rods which did not produce any clearing on uric acid plates. Previously all cultures had been shown to possess no urease activity.

The organisms were grown in a uric acid liquid medium plus

0.25% Difco yeast extract for 7 days at 22°. After this incubation no ammonia could be detected in any of the cultures when tested with Nessler's reagent. To 1 ml. of culture was added 5 drops of a 0.016% phenol red solution, the pH was adjusted to approximately 7.0 with 0.02N-CH_3COOH and 1 ml. of a freshly prepared 1% soy bean meal suspension was added. The tubes were shaken and kept at 45° for 2 hours. A control of uninoculated medium was similarly treated.

Soy bean contains the enzyme urease which converts any urea present to ammonia and carbon dioxide. This results in a rise in pH shown by a colour change in the indicator from yellow to red. Tubes showing a red colour were therefore taken as urea-positive.

It was found that the organisms which produced clearing on uric acid agar formed urea as a degradation product. No ammonia was formed. No urea or ammonia was detected in the uninoculated control tube or in those cultures which failed to produce clearing on uric acid agar.

Sometimes with old cultures of organisms which produced clearing on uric acid agar, but which were urease-negative, a slightly more dense precipitate appeared at the edge of the zone of clearing. This failed to give a positive reaction with Nessler's reagent and hence was not ammonium urate although similar in appearance. Microscopic examination of the crystals showed fan-shaped and sheath-like clusters identical with the

form of the crystals of sodium hydrogen urate. It is not known why the acid urate should crystallize out. It may be connected with pH as the same effect can be observed if a drop of N-NaOH is added to the centre of a uric acid plate. At the edge of the clearing where the uric acid dissolves a precipitate of sodium hydrogen urate forms.

Decomposition of urea to ammonia

For the detection of urea decomposition a medium of the following composition was used: Meat extract (Lab-Lemco), 1 g.; peptone, 1 g. in 100 ml. water at pH 7.0. To this was added filter-sterilized urea to give a final concentration of 2% (w/v). Cresol red (concn. 0.0016%) was included to indicate the rise in pH associated with urea hydrolysis. This indicator was chosen in preference to phenol red, as used by Rustigian & Stuart (1941), because of its higher pH range. In this way only organisms having appreciable activity were detected.

The medium was dispensed aseptically into tubes in 5 ml. quantities. Cultures were incubated for 7 days at 22°. At the end of this period cultures showing a bright red colour were recorded as positive.

Positive cultures were titrated with acid according to Cunningham's (1947) method. To 1 ml. of culture was added 10 ml. of water and 1 drop of methyl red indicator solution (0.08%). This was titrated with N/14-H_2SO_4 until the colour just began to change. The solution was boiled to expel carbon dioxide and

the titration completed while the liquid was still hot. Each ml. of acid used is equivalent to 1 mg. of ammoniacal nitrogen.

The titration figures recorded in this work have not been corrected for any slight alkalinity which might have been produced from the broth medium itself.

Plate count for aerobic uric acid decomposers

As uric acid-decomposing organisms had been shown to produce clearing around their colonies on a uric acid agar plate the possibility existed that counts of such organisms might be made by employing a surface plating technique.

Plates were therefore prepared as previously described consisting of the uric acid medium plus 0.25% Difco yeast extract. A 1% suspension of uric acid was added to the top layer of agar. The plates were dried and 0.1 ml. of successive ten-fold dilutions of the litter were spread over the surface. Plates were incubated for 5 days at 22°. Longer periods of incubation caused merging of the cleared zones resulting in difficulty in counting. After incubation the plates were flooded with N-H_2SO_4 in case any false zones of clearing existed. The number of colonies showing clearing were counted and an estimate was made of the total number of aerobic uric acid decomposers in the sample.

The results for a number of samples of built-up litter are given in Table 10, expressed in terms of the dry weight. When these counts are expressed as percentages of the corresponding

Table 10

Plate counts for aerobic uric acid decomposers of samples of built-up litter

Sample no.	Plate counts x 10^{-6}/g. Dry weight	As % of total count	Uric acid pos. orgs. on total count plates %	Ratio of percentages
4b	2,300	5.1	14	0.36
4c	3,900	9.0	32	0.28
4d	2,300	4.7	16	0.29
4e	5,200	8.5	34	0.25
4f	923	4.6	21	0.22
5	6,300	6.9	28	0.25
7	2,100	3.3	16	0.21

total counts (also in terms of the dry weight) they give lower figures than would be expected from the percentages of colonies picked from the total count plates which proved on testing to be uric acid decomposers. This means that the plating method is not detecting all the uric acid decomposers present. This is probably due to the fact that some of the uric acid decomposers are far more active than others. If the plates were to be incubated longer than five days weakly active strains would produce zones of clearing but by this time the more active strains would have completely cleared the plate.

Although all uric acid decomposers are not counted by this method the fraction detected is roughly constant as shown by the last column of figures. Thus counts obtained by this method will give some indication of the numbers of uric acid decomposers present.

Except for sample 4f, the numbers of aerobic uric acid decomposers in the built-up litter samples examined were all of the same order of magnitude. Litter 4f had a slightly lower count but the total count of this sample was also correspondingly less (Table 3). This was probably due to the fact that the temperature of this sample had dropped to 8° and bacterial activity would have been low.

Types of micro-organisms capable of decomposing uric acid

From poultry litter and droppings were isolated 224 organisms capable of decomposing uric acid. Some of these

also show urease activity and form ammonia in the decomposition of uric acid. The distribution of these organisms into genera is shown in Table 11; more detailed descriptions within each genus are given in Part III.

The figures given in Table 11 do not represent the relative numbers of the different types in poultry litter as most of the Gram-negative organisms were obtained by selective means. Obviously, however, corynebacteria are the most important types concerned in the breakdown of uric acid in poultry litter.

The decompostion of uric acid by strains of corynebacteria, nocardia and pseudomonads has previously been reported. No reference has been found, however, to members of the genera Streptomyces, Alcaligenes and Achromobacter taking part either in this reaction or in the hydrolysis of urea to ammonia.

Den Dooren de Jong (1927) described a Gram-negative organism, Protaminobacter alboflavum α, which was able to utilize uric acid as an energy source. Organisms belonging to this genus are defined as being capable of dissimilating alkylamines. Gray & Thornton's (1928) medium, with the nitrogen source omitted, was used for testing the ability of the Gram-negative uric acid decomposers to utilize certain simple amines. This had the following composition: K_2HPO_4, 1.0 g.; $MgSO_4.7H_2O$, 0.2 g.; NaCl, 0.1 g.; $CaCl_2$, 0.1 g.; $FeCl_3.6H_2O$, 0.02 g.; distilled water to 1000 ml.; pH 7.0. Methylamine and ethylamine were sterilized separately as 1%

Table 11

Types of micro-organisms capable of decomposing uric acid

	No. of strains	No. possessing urease activity
Corynebacterium	134	10
Nocardia	3	0
Streptomyces	4	4
Pseudomonas	28	0
Alcaligenes	37	8
Achromobacter	18	1

solutions and added aseptically to give a final concentration of 0.2% (w/v). All the isolates tested failed to utilize either methylamine or ethylamine and hence are unlike Protaminobacter alboflavum ∝.

Types of micro-organisms capable of decomposing urea but not uric acid

Eighty-six isolates were found which decompose urea but not uric acid. These belong to the following genera and are discussed in greater detail in Part III:

Corynebacterium, 22 strains

Micrococcus, 17 strains

Alcaligenes, 8 strains

Achromobacter, 20 strains

Cytophaga, 8 strains

Urease activity has previously been reported for certain strains of Corynebacterium and Micrococcus. As already stated in connection with the breakdown of uric acid, urease activity in the genera Alcaligenes and Achromobacter is not a commonly reported characteristic. This also applies to the genus Cytophaga whose activities, apart from cellulose decomposition, have not been widely studied.

The anaerobic decomposition of uric acid

Several samples of poultry litter were examined for anaerobic uric acid decomposers using a tube dilution method. The medium used was that of Barker & Beck (1942) and contained

uric acid, 5.0 g.; yeast autolysate, 5 ml.; K_2HPO_4, 0.3 g.; $MgSO_4.7H_2O$, 0.1 g.; phenol red indicator solution (1.6% alcoholic), 1 ml.; distilled water to 1000 ml. The ingredients were suspended in almost the whole volume of water, the mixture brought to the boil and the pH adjusted to 7.2-7.6 by addition of N-NaOH. The medium was made up to 1 litre and sterilized in 100 ml. quantities at 15 lb. for 15 min. Before use the medium was boiled and allowed to cool. It was found advisable to use this medium as soon as possible after preparation as crystallization occurred after a few weeks. The crystals were microscopically similar to those of the acid urate (sodium hydrogen urate) and could not be redissolved by boiling.

Samples for examination were macerated in the normal way to give a 1/10 suspension. From this, ten-fold dilutions were made up to $1/10^6$. One ml. quantities of each dilution, in duplicate, were placed in 6" x ¾" test tubes. The tubes were filled with medium, tightly stoppered with rubber bungs and incubated at 30^o for 7 days.

Growth usually was apparent within 48 hours in positive tubes. With the higher dilutions growth sometimes did not appear until after 4 days but no positive tubes ever appeared after this time. Growth was accompanied by a rise in alkalinity and at first a heavy precipitate of ammonium urate which later disappeared as the decomposition proceeded. No gas was formed. Higher dilutions sometimes showed a very slight cloudiness with

no change in pH. This was found to be due to the growth of corynebacteria indicating that conditions were not absolutely anaerobic. Such tubes were considered negative.

Microscopic examination of positive tubes revealed the presence of spore-forming clostridial types. The extent to which these organisms occurred in the litter samples examined is shown in Table 12.

In no case were anaerobic uric acid decomposers detected in less than 0.001 ml.; that is, their numbers were less than 10,000 /g. of material. This number is very small when compared with the number of organisms generally present capable of decomposing uric acid aerobically. In consequence they are probably of little significance in the decomposition of uric acid in built-up litter and their study was not pursued.

No attempt was made to isolate and identify the organisms concerned but morphologically they resembled Clostridium acidi-urici (Liebert) Barker & Beck (Barker & Beck, 1942). In smears from positive tubes they appeared as weakly Gram-positive rods with oval, terminal spores producing distension of the rod. No anaerobic cocci were detected in any of the positive tubes.

Table 12

The occurrence of anaerobic uric acid decomposers in poultry litter

Sample no.	Description of litter	Highest dilution showing positive growth
1a	Chopped straw and peat moss; before chicks introduced	No anaerobic uric acid decomposers detected
1b	Same litter as above; age 3 months	$1/10^3$
1c	Same litter as above; age 9 months (very wet)	$1/10^3$
2	Composition not known; age 2 months	$1/10^3$

SUMMARY

An agar medium containing uric acid in suspension was used for the detection of aerobic uric acid decomposers. The possibility of using this medium for the enumeration of uric acid decomposers was also explored. The results were not entirely satisfactory, the count being on an average, only approximately one-quarter of the total number of uric acid decomposers.

The ability to hydrolyse urea was determined in urea broth containing cresol red as an indicator. A more quantitative estimation of urease activity was obtained by titration of the urea broth culture with acid.

Most organisms capable of decomposing uric acid aerobically form urea as an end-product; a few, however, are capable of forming ammonia. Strains of Corynebacterium are the most important decomposers of uric acid. Other organisms having this ability belong to the genera Nocardia, Streptomyces, Pseudomonas, Alcaligenes and Achromobacter.

Urease activity was displayed by strains of Corynebacterium, Micrococcus, Alcaligenes, Achromobacter and Cytophaga.

By employing an anaerobic uric acid medium, spore-formers morphologically resembling Clostridium acidi-urici were detected. These organisms form ammonia in the decomposition process. In comparison with the number of aerobic uric acid decomposers, the number of these clostridia was very small.

As a result of this investigation it may be concluded that the decomposition of uric acid in built-up poultry litter is brought about mainly by the activities of aerobic organisms. Decomposition results in the formation of ammonia.

PART III

A SYSTEMATIC STUDY OF MICRO-ORGANISMS FROM POULTRY LITTER AND DROPPINGS

A SYSTEMATIC STUDY OF MICRO-ORGANISMS FROM POULTRY LITTER AND DROPPINGS

INTRODUCTION

In the following section are described those isolates from poultry litter and droppings which were found to be of particular interest. Many of these are concerned in the production of ammonia from uric acid and are probably responsible for the ammoniacal smell and high alkalinity of built-up poultry litter. Others have been shown to be active synthesizers of substances having vitamin B12 activity. These are of interest in connection with the occurrence of this vitamin in built-up poultry litter.

The organisms described belong to the genera Corynebacterium, Nocardia, Streptomyces, Micrococcus, Pseudomonas, Alcaligenes, Achromobacter, Flavobacterium and Cytophaga. Their characteristics and possible identity are discussed.

EXPERIMENTAL METHODS

Source of cultures

(a) Isolates from poultry litter and droppings

The isolates examined originated mainly from high dilution plates used for estimating the total and Gram-negative counts of poultry litter and droppings. A few were isolated from uric acid agar plates and from enrichment cultures in a liquid uric acid medium. Before being examined in detail all cultures were purified by replating.

(b) Named cultures for comparison

Bacterium ammoniagenes

NCTC 2398.

Bacterium globiforme

Originally obtained from Prof. H. J. Conn.

Bacterium linens

NCIB 8546.

Corynebacterium helvolum

NCIB 8605. Strain Jensen 163.

Corynebacterium ovis

NCTC 3450. Strain E 23 (ovine).

Corynebacterium renale

NCTC 7448. Strain Charita - a.

Corynebacterium ureafaciens

NCIB 7811. Identical with Corynebacterium creatinovorans. Strains 81 and 178 isolated by Topping (1936).

(NCTC, National Collection of Type Cultures; NCIB, National Collection of Industrial Bacteria).

Media and methods used for examination of isolates and named cultures

Unless otherwise stated cultures of micrococci were incubated at 30° and those of other organisms at 22°.

Morphology and motility. Cultures were inoculated on to the surface of nutrient agar or peptone-yeast extract agar slopes and incubated at 22°. As soon as growth appeared, films were prepared and examined by Gram's method. Cultures were again examined after further incubation periods of 24 hr. and 48 hr. and then at less frequent intervals up to 14 days. The condensation water at the base of the slope was used for testing motility. Flagella were demonstrated by the Casares-Gil staining method (Thatcher, 1926).

Cultural characteristics on agar and in broth. For the majority of strains nutrient agar and nutrient broth were used. A few cultures grew poorly or not at all in these media. For these, Topping's (1936) medium was used consisting of peptone, 0.25 g.; yeast extract, 0.25 g.; water to 100 ml.; pH 7.0. Cultures were incubated at 22° and examined after 7 and 14 days.

Cultural characteristics in glucose agar. Stab inoculations were made in glucose agar containing brom-cresol purple (concn. 0.0016%) as an indicator. This medium consisted of glucose, 0.5 g.; peptone, 0.5 g.; meat extract (Lab-Lemco), 0.5 g.; NaCl, 0.25 g.; agar, 1.5 g.; water to 100 ml.; pH 7.0.

These cultures were used for determining the oxygen requirements of the organisms; pH changes were noted up to 10 days.

Temperature requirements for growth. Agar slopes were inoculated from a broth culture of the organism and incubated at 22°, 30° and 37° for 7, 4 and 2 days respectively.

Gelatin liquefaction. Stab inoculations were made in 12% (w/v) nutrient gelatin; cultures were incubated for 28 days at 22°.

Haemolysis. Cultures were streaked on nutrient agar containing 5% (v/v) of ox blood; these were incubated for 7 days.

Salt tolerance. Double strength peptone-yeast extract broth was tubed in 2.5 ml. quantities. To each tube was added, aseptically, an equal volume of double strength NaCl solution so as to give final concentrations of 5, 10 and 15% (w/v) of NaCl. A loopful of a light suspension in saline was used for inoculation; growth was recorded after 14 days.

Catalase test. 'Five vol.' hydrogen peroxide was run down the surface of a 24 hr. slope culture. The production of gas indicated a positive reaction.

Coagulase test. This was carried out by adding 1 ml. of an 18 hr. broth culture to 1 ml. of a 1/10 dilution of human plasma in saline. The tubes were examined after incubation at 37° for 4 hr. and again after standing overnight at room temperature.

Carbohydrate fermentation. The basal solution used contained peptone, 1.0 g.; NaCl, 0.5 g.; brom-cresol purple (concn.

0.0016%) as an indicator; water to 100 ml.; pH 7.0. The carbohydrate was autoclaved separately and added aseptically to give a concn. of 0.5% (w/v). Final readings were made after 14 days.

Action on glucose. Organisms were grown in glucose peptone water (as used for glucose fermentation but without indicator) for 7 days and tested for the presence of acetoin using Barritt's (1936) modification of the Voges-Proskauer test. A methyl red test was also made on these cultures.

Diastatic action. The peptone-yeast extract agar medium plus 1% (w/v) of soluble starch was used for the detection of diastatic activity. Streak cultures were made on the surface of poured plates, one plate being used for each strain. After incubation for 10 days the plates were flooded with iodine solution; absence of the blue colour in the vicinity of growth indicated a positive reaction.

Action on organic acids. The basal medium consisted of NaCl, 0.5 g.; $MgSO_4.7H_2O$, 0.02 g.; $NH_4H_2PO_4$, 0.1 g.; K_2HPO_4, 0.1 g.; Difco yeast extract, 0.05 g.; agar, 1.5 g.; water to 100 ml.; pH 6.8. An aqueous solution of brom-thymol blue was added to give a final concentration of 0.0016%. The organic acids tested included acetic, citric, succinic, gluconic, lactic, benzoic, oxalic and propionic; these were added to give a concn. of 0.2% (w/v). Light suspensions of the organisms were made in saline and a loopful spread over the surface of each of the media

sloped in tubes. These were examined daily up to 14 days. The production of growth in excess of that produced in a control tube containing no added organic acid was taken as indicating utilization of the acid concerned; changes in the reaction of the medium were also recorded.

Litmus milk. Cultures in litmus milk were incubated for 28 days and examined frequently.

Uric acid decomposition. The medium used for detecting uric acid decomposition is described on p. 30; to this was added 0.25% of Difco yeast extract.

Urea hydrolysis. The medium used for detecting urea hydrolysis is described on p. 35.

Nitrate reduction. Organisms were grown in nitrate peptone water (peptone, 1.0 g.; NaCl, 0.5 g.; KNO_3, 0.1 g.; water to 100 ml.; pH 7.0) and tested by the addition of Ilosvay reagents. Micrococci were tested after 7 days; other organisms after 14 days.

Action on thymine, uracil, creatine and creatinine. The basal medium consisted of NaCl, 0.5 g.; $MgSO_4.7H_2O$, 0.02 g.; K_2HPO_4, 0.25 g.; Difco yeast extract, 0.05 g.; water to 100 ml.; pH 6.8. This medium, to which 0.2% (w/v) of the test compound was added, was dispensed in 5 ml. quantities in tubes. Cultures were incubated for 14 days and then tested for ammonia using Nessler's reagent and for urea by the method described on p. 33.

Utilization of simple nitrogenous compounds. The following

media were used. Growth was recorded after 14 days.

(a) Glucose-NH_4^+ medium. This medium consisted of NaCl, 0.5 g.; $MgSO_4.7H_2O$, 0.02 g.; $NH_4H_2PO_4$, 0.1 g.; K_2HPO_4, 0.1 g.; water to 100 ml.; pH 6.8. Brom-thymol blue (concn. 0.0016%) was added as an indicator. This was sterilized in 5 ml. quantities in tubes and a 10% glucose solution added aseptically to give a final concn. of 0.02% (w/v).

(b) Glucose-NO_3^- medium. This medium consisted of NaCl, 0.5 g.; $MgSO_4.7H_2O$, 0.02 g.; K_2HPO_4, 0.25 g.; $NaNO_3$, 0.1 g.; water to 100 ml.; pH 6.8. Brom-thymol blue (concn. 0.0016%) was added as an indicator. This was sterilized in 5 ml. quantities in tubes and a 10% glucose solution added aseptically to give a final concn. of 0.02% (w/v).

(c) Glucose-urea medium. This medium consisted of NaCl, 0.5 g.; $MgSO_4.7H_2O$, 0.02 g.; K_2HPO_4, 0.25 g.; water to 100 ml.; pH 6.8. Brom-thymol blue (concn. 0.0016%) was added as an indicator. This was sterilized in 5 ml. quantities in tubes, to each was added glucose as in (a) and (b) and then 5% urea solution (filter sterilized) to give a final concn. of 0.1% (w/v).

Citrate-NH_4^+ medium. This medium consisted of NaCl, 0.5 g.; $MgSO_4.7H_2O$, 0.02 g.; $NH_4H_2PO_4$, 0.1 g.; K_2HPO_4, 0.1 g.; Na citrate, 0.2 g.; water to 100 ml.; pH 6.8. Brom-thymol blue (concn. 0.0016%) was added as an indicator and the medium was sterilized in 5 ml. quantities in tubes. Growth was

recorded after 14 days.

Vitamin B12 production. The method used for detecting vitamin B12 production is described on p. 120.

Additional tests for which no positive cultures were found

Action on cellulose. A strip of filter paper added to a tube of peptone-yeast extract broth was used for detecting cellulolytic action. Tubes were examined weekly for 6 weeks.

Growth with naphthalene. Representative cultures were streaked on the surface of poured agar plates of the following composition: NaCl, 0.5 g.; $MgSO_4.7H_2O$, 0.02 g.; K_2HPO_4, 0.25 g.; NH_4NO_3, 0.1 g.; agar, 1.5 g.; water to 100 ml.; pH 6.8. Cultures were inverted over naphthalene placed in the lids of the Petri dishes and incubated for 14 days.

Growth with cholesterol. Representative cultures were streaked on the surface of agar slopes of the following composition: NaCl, 0.5 g.; $MgSO_4.7H_2O$, 0.02 g.; K_2HPO_4, 0.25 g.; NH_4NO_3, 0.1 g.; cholesterol, 0.1 g.; agar, 1.5 g.; water to 100 ml.; pH 6.8. Tubes were incubated for 14 days.

Growth with nicotine. Representative strains were tested on the medium of Sguros (1955); growth was recorded after 14 days.

EXPERIMENTAL AND RESULTS

Corynebacterium

The strains studied were shown to have characteristics which clearly ally them with the soil corynebacteria. They are all strict aerobes.

In young cultures they appear as Gram-positive cocco-bacilli to moderately long rods, the size of the cells varying considerably from strain to strain. Even with one strain cell size is not a constant feature, the cells decreasing in length and becoming cocco-bacillary as the culture ages. Accompanied by this morphological change is a tendency for the cells to be less intensely Gram-positive, although this does not always occur. Young cultures show the angular and palisade arrangement of cells generally associated with the coryneform bacteria. As the cells become coccal this typical arrangement is lost and in old cultures irregular groups of cells are seen. It is therefore possible for cultures of corynebacteria to appear exactly as micrococci, a phenomenon which stresses the importance of examining corynebacteria for morphology at an early stage of growth. Some strains show active motility. None form spores.

Particular attention was given to the study of those strains which, in some way, are concerned in the decomposition of uric acid. Most of the strains capable of attacking uric acid form urea but a few carry the reaction still further and

form ammonia. Other strains were found which do not attack uric acid but actively hydrolyse urea.

Attention has also been paid to a group of corynebacteria which were found to be active synthesizers of vitamin B12-active compounds.

Corynebacteria capable of decomposing uric acid

The number of strains examined was 134. This represents 43% of the total number of corynebacteria examined. As these corynebacteria had been picked at random from high dilution plates used for estimating the total count it means that, on an average, about half the corynebacteria in built-up poultry litter are uric acid decomposers.

It was not possible to divide the strains into clearly defined types. Their characteristics were found to form a continuous spectrum; the strains at one end are weakly proteolytic and form acid from glucose, the strains at the other end are strongly proteolytic and do not form acid from glucose.

For purposes of description and for comparison with certain named cultures the strains have been divided into three groups the characteristics of which are given in Table 13. These particular characters were chosen because they have been used frequently in the past for describing and differentiating coryneform bacteria of soil origin.

Apart from the differences noted in Table 13 the strains

Table 13

Characters of the strains of Corynebacterium capable of decomposing uric acid

	A	B	C
No. of cultures	49	75	10
Reaction in glucose agar	Slight to moderate acidity	Neutral or alkaline	Neutral or alkaline
Diastase production	+	11+ 64-	-
Litmus milk	44 no change; 5 rennet clot, litmus reduced, no proteolysis	Rapid proteolysis, litmus reduced, often alkaline and clotted	Proteolysis, litmus reduced, often alkaline, may be clotted
Gelatin liquefaction	Slight; few strains negative	+	8+ 2-
Urea hydrolysis	-	-	+
Utilization of N compounds			
NH_4^+	-	Variable	-
NO_3^-	-	Variable	-
Pigment on agar	None, cream or lemon-yellow	Pale orange to orange, a few cream, yellow or none	Pale to bright orange, one yellow
Growth at 37°	11+ 38-	22+ 53-	-
Motility	-	-	1+ 9-

also show a gradual transition in cell size and in size of colony on agar. Group A strains appear in young culture as rods, 0.8-1 x 1-3μ; the diastase-positive strains of group B are larger (1 x 1.5-4μ) and the diastase-negative strains of groups B and C are still larger in size (1-1.2 x 2-8μ). Colony size also increases in the same way with a tendency for pigment production to be orange rather than to be absent, cream or yellow. However none of these characteristics are definite enough to warrant their use for differential purposes.

The ability of group A strains to produce acidity in glucose agar is also a doubtful test when considered alone. In glucose agar some of the diastase-positive strains placed in group B produce a transient acidity. Smaller or larger amounts of acid are also formed when the constitution of the medium used for the test, is varied.

Corynebacteria capable of decomposing uric acid were isolated from all samples of built-up litter examined and also from the sample of poultry droppings. The types found appear to be independent of the composition of the litter, its age, moisture content, pH or temperature.

All strains grow well at 30°. Strains capable of growth at 37° under laboratory conditions did not originate especially from built-up litter samples which were, or had been, well heated. As the temperature of built-up litter in Scotland was never found to exceed 30°, the selection of strains capable

of growth at 37° probably does not occur.

Several named cultures of coryneform bacteria were found to be capable of decomposing uric acid. These are Bacterium globiforme, Corynebacterium helvolum, Bacterium linens, Corynebacterium ureafaciens and Topping's two strains 81 and 178. Other characteristics are shown in Table 14.

Bacterium globiforme and Corynebacterium helvolum were found to be very similar to each other, differing only slightly in morphology. Initially they produce acidity in glucose agar; they are diastase-positive, weakly proteolytic in milk and produce a pale cream pigment. In these characteristics they resemble the isolates from poultry litter placed in group A and also the diastase-positive strains of group B. There is possibly little justification for having these two organisms as distinct species, as in Conn & Dimmick's (1947) classification for Arthrobacter, especially as C. helvolum no longer produces a lemon-yellow pigment.

The production of a lemon-yellow pigment by many of the group A isolates is of no use as a differential character. Yellow chromogenic forms of the Bact. globiforme group have frequently been isolated from soil (Taylor & Lochhead, 1937; Conn & Dimmich, 1948; Lochhead, 1948). The pigment has been found to vary in intensity and may disappear on artificial culture. It seems preferable to consider these chromogenic strains as variants of Bact. globiforme as suggested by

Table 14

Characters of the named coryneform bacteria capable of decomposing uric acid

	Bact. globiforme C. helvolum	Bact. linens	Topping's cultures 81 and 178	C. ureafaciens
Reaction in glucose agar	Slightly acid, becoming slightly alkaline	Alkaline	Slightly acid	Slightly acid, becoming slightly alkaline
Diastase production	+	-	-	-
Litmus milk	Alkaline, slow digestion	Alkaline, slow digestion	Alkaline, clot, reduction, strong peptonization	Alkaline, reduction, strong peptonization
Gelatin liquefaction	Slight	+	+	+
Urea hydrolysis	-	-	-	-
Utilization of N compounds NH_4^+) NO_3^-)	Variable	-	-	Variable
Pigment on agar	Pale cream	Bright orange	Pale cream	Lemon-yellow
Growth at 37°	+	-	-	+
Motility	-	-	+	-

Lochhead (1955).

It is possible that the diastase-negative strains of group B are similar to Arthrobacter simplum (Conn & Dimmick, 1948). This organism is described as being similar to Arthrobacter globiforme but having no diastatic action on starch.

Bacterium linens is more closely related to the group B strains. It fails to produce acidity in glucose agar and is diastase-negative. It produces an orange pigment which was also found to be a common feature of the diastase-negative strains of group B. It produces angularly arranged rods in young culture which become shorter and finally almost coccal in old cultures. Because of its similarity to Bact. globiforme and C. helvolum there appears to be no sound reason for separating it in a different genus, that of Brevibacterium, as proposed by Breed (1953). The particular organism examined appears to be quite typical of the strains of this species which have been isolated from cheese (Kelly, 1937; Kelly & Marquardt, 1939; Yale, 1943; Albert, Long & Hammer, 1944).

Salt tolerance has been reported (Kelly & Marquardt, 1939; Albert, Long & Hammer, 1944) as being a characteristic feature of Bact. linens. It was found that this particular strain is able to grow in the presence of 10% of salt but so also can most of the corynebacteria isolated from poultry litter and droppings. Some of these isolated strains also

produce slight growth in the presence of 15% of salt. This characteristic is therefore of no use for differentiating Bact. linens from other coryneform bacteria.

Corynebacterium ureafaciens and one of Topping's cultures, namely 178, were recently studied by Clark (1955). The former organism was first isolated by Dubos & Miller (1937) who described it as being a small pleomorphic Gram-positive aerobic bacterium which could utilize creatine or creatinine as its sole source of carbon and nitrogen, producing urea as an end-product. The organism grew well on common media with the production of a yellow pigment. No attempt was made to identify the organism and it was simply designated as culture NC. According to Clark (1955) the culture was later sent to the American Type Culture Collection under the name Corynebacterium creatinovorans. This same organism was further examined by Krebs & Eggleston (1939) who assigned to it the name Corynebacterium ureafaciens. These two names are therefore synonymous. Krebs & Eggleston confirmed Dubos & Miller's earlier description and in addition stated that the organism was non-motile, could use ammoniacal nitrogen, oxidised but did not ferment carbohydrates, failed to reduce nitrate, liquefied gelatin and did not produce urease.

In addition to C. ureafaciens and Topping's culture 178 Clark (1955) also examined three cultures labelled Arthrobacter aurescens which he had received from Prof. H. J. Conn. These

five cultures were capable of growing on either creatine, creatinine or uric acid as a sole source of carbon and nitrogen and were urease-negative. All produced a yellow pigmentation varying in intensity. Clark proposed that these cultures should be placed in the genus Arthrobacter and given the name Arthrobacter ureafaciens (Krebs & Eggleston) comb. nov.

When C. ureafaciens and Topping's cultures 81 and 178 were examined during the present investigation, they were found to be very similar to the group B strains; slight acidity is initially produced in glucose agar, starch is not attacked and litmus milk is rapidly digested. The three cultures are therefore closely related to Bact. globiforme and C. helvolum. It is regrettable that Clark did not also include these two latter organisms in his study because they also will decompose creatine, creatinine and uric acid if a heavy inoculum is used or if a little yeast extract is added to the medium.

The strongly proteolytic members of group B which produce an alkaline reaction in sugar media and which are diastase-negative appear to be similar to the Corynebacterium filamentosum-simplex group of Jensen (1934).

Strains placed in group C are urease-positive as shown by their reaction in urea broth containing cresol red. They form ammonia as a result of uric acid decomposition. It is probable that there exist also in poultry litter strains of corynebacteria which decompose uric acid but whose urease activity is so weak

that they fail to change the colour of cresol red in urea broth cultures. Such was found to be the case with C. helvolum and Topping's culture 81; these show ammonium urate crystals on uric acid agar plates but display urease activity in urea broth only if the cresol red is replaced by phenol red which changes colour at a lower pH.

One strain placed in group C is of particular interest in that it is capable of producing an excess of vitamin B12-active compounds; this strain is also capable of decomposing uracil and thymine forming ammonia. Another strain placed in group C shows active motility. This is by no means a rare characteristic in the coryneform group. Topping's cultures 81 and 178 belong to her group of motile Gram-positive pleomorphic rods from soil (Topping, 1936). Motility has been noted in cultures, designated as Bact. globiforme by Taylor (1938) and by Lochhead (1940). Clark & Carr (1951) have also reported motility in cultures of certain soil coryneform bacteria. It would be unreasonable, therefore, to separate off any organisms, which otherwise show similar characteristics, on the basis of motility.

Accessory growth factors necessary for uric acid decomposition. The decomposition of uric acid by representative strains of the isolated corynebacteria and by the named coryneform organisms was studied in greater detail. This was done by streaking the organisms on the surface of uric acid agar plates to which

various additions had been made. The plates were incubated at 22° for 14 days and the results for uric acid decomposition, as evidenced by clearing, are shown in Table 15. These results show that certain of the organisms fail to decompose uric acid unless provided with an additional nitrogen source such as peptone or yeast extract. Yeast extract supplies more effectively than does peptone the factor(s) necessary for growth. Some of the strains which decompose uric acid with no additional nitrogen compound fail to do so if a liquid medium with only a very small inoculum is used. This applies to Bact. globiforme and C. helvolum. It therefore seems advisable, if considering the decomposition of uric acid as a differential test, to add yeast extract to the medium so that variable results, arising from differences in the size of inoculum, are not obtained. This point is well illustrated by considering Topping's strain 178. Clark (1955) states that this organism can grow on uric acid as the sole source of carbon and nitrogen whereas in the present work (Table 15) the addition of peptone or yeast extract was found to be necessary for utilization of this compound.

Further characters of the coryneform bacteria capable of decomposing uric acid. Further examination of representatives from all the corynebacteria isolated, revealed that the ability to decompose creatine and creatinine, with the production of ammonia and urea, is confined to those strains capable of decomposing uric acid. The results for the strains tested and

Table 15

Accessory growth factors necessary for uric acid decomposition

Representatives of the isolated strains	Uric acid agar				
	with no addition	plus 0.2% glucose	plus 0.2% peptone	plus 0.05% yeast extract	plus 0.2% yeast extract
Grp. A 6-10	-	-	+/-	-	++
3-8	-	-	+	++	++
11-23	-	-	-	+/-	++
12-17	-	-	-	-	++
Grp. B 10-21	+	+	++	++	++
8-18	+	+	++	++	++
12-15	+	+	++	++	++
11-10	+	+	++	++	++
11-19	+	+	++	++	++
Grp. C 8-7	+	+	++	++	++
15-8	+	+	++	++	++
7A-24	+	+	++	++	++
Bact. globiforme	++	++	++	++	++
C. helvolum	++	++	++	++	++
Topping's cultures					
81	-	-	+	++	++
178	-	-	++	++	++
C. ureafaciens	++	++	++	++	++
Bact. linens	-	-	++	++	++

Decomposition is assessed by the number of positive signs.

for the named cultures are shown in Table 16. Included also in this table are the results for organic acid decomposition. The ability to decompose creatine and creatinine does not help in subdividing the uric acid decomposers isolated from poultry litter, positive strains being found in each of the groups A, B and C. The results for the decomposition of organic acids again show the transition in characters between strains placed in group A and those in groups B and C. Bact. globiforme and C. helvolum again resemble most closely the group A strains; Topping's culture 81, C. ureafaciens and Bact. linens the group B strains. Topping's culture 178, although resembling the group B strains in other respects, has little action on organic acids; it also failed to decompose creatine and creatinine in contrast with Clark's (1955) finding.

Discussion and conclusions. The results indicate that no sharp divisions occur in the characteristics of the coryneform bacteria capable of decomposing uric acid. Those isolated from poultry litter and droppings appear to be closely related to the Bacterium globiforme group normally present in soil. In built-up litter growth of these organisms would be enhanced by the continual addition of uric acid in the faeces of the birds. Decomposition of the uric acid by these organisms would result in the formation of urea and ammonia.

Corynebacteria capable of hydrolysing urea

These organisms are capable of hydrolysing urea but have

Table 16

Further characters of the coryneform bacteria capable of decomposing uric acid

Representatives of the isolated strains	Decomposition of: Acetate	Citrate	Succinate	Gluconate	Lactate	Propionate	Creatine Creatinine
Grp. A 6-10	-	-	-	+	-	-	-
3-8	-	-	-	-	-	-	+
11-23	+K	-	-	+	+K	+K	-
12-17	-	-	-	+	+K	+sl.K	-
Grp. B 10-21	+K	+K	+K	+sl.K	+K	+K	+
8-18	+K	+K	+K	+sl.K	+K	+K	+
12-15	+K	+K	+K	+sl.K	+K	+K	+
11-10	+K	+K	+K	+sl.K	+K	+K	+
11-19	+K	+K	+K	+sl.K	+K	+K	-
Grp. C 8-7	+K	+K	+K	+sl.K	+K	+K	+
15-8	+K	+K	+K	+sl.K	+K	+K	-
7A-24	+K	+K	+K	+K	+K	+K	+
Bact. globiforme	-	-	-	-	-	-	+
C. helvolum	+K	+sl.K	-	-	+K	-	+
Topping's cultures							
81	+K	+K	+K	+sl.K	+K	+K	+
178	-	+sl.K	-	-	-	-	-
C. ureafaciens	+K	+K	+K	-	+K	+K	+
Bact. linens	+K	+K	+K	+sl.K	+K	+K	-

K = alkaline reaction; sl. = slight.

no action on uric acid. Twenty-two strains were examined; they originated from both built-up poultry litter and from droppings.

Urease activity has been reported for several of the corynebacteria of animal origin; namely Corynebacterium renale (Lovell, 1946; Lovell & Harvey, 1950; Morse, 1948), Corynebacterium ovis (Lovell & Harvey, 1950; Morse, 1949) and Bacterium ammoniagenes, a Gram-positive coryneform organism isolated by Cooke & Keith (1927) from the faeces of infants. Cultures of these three species were obtained for comparison; their urease activity was confirmed and they proved to have no action on uric acid.

The isolated strains and Bact. ammoniagenes occur in young culture as angularly arranged Gram-positive rods, 0.8-1 x 2-4μ, which become more coccal in older culture. C. renale is a smaller rod with less tendency to become cocco-bacillary; C. ovis occurs even in young culture as a small cocco-bacillus. All are non-motile. Colonies of the isolated strains on agar are circular, 1-2 mm. in diameter, low convex, smooth, glistening, entire, opaque and butyrous. Bact. ammoniagenes and C. renale have similar colonies to those of the isolated strains; the colonies of C. ovis differ in being umbonate, dull and slightly friable. All strains produce acidity at the surface of a glucose agar stab culture; in this respect they differ from Corynebacterium pseudodiphtheriticum (Hofmann's bacillus), a urease-positive organism

but which has no action on sugars. None of the organisms utilizes inorganic nitrogen.

Further characteristics of the organisms examined are shown in Table 17. C. renale is distinct in producing an alkaline reaction in litmus milk; it also actively clears nutrient agar containing 10% milk. The strain of C. ovis examined is haemolytic and differs markedly morphologically from the other organisms examined.

All the isolated strains liquefy gelatin but as the action, in many cases, is only slight this property cannot be used as a definite distinguishing feature between these strains and Bact. ammoniagenes. Some of the isolated strains are diastase-positive but the distinction between some of these and the diastase-negative strains is not definite; a few strains produce a rennet clot in milk. It is not considered, however, that the strains concerned should be separated on these characteristics and all the isolated strains are regarded as belonging to the same species as Bact. ammoniagenes. The ability of Bact. ammoniagenes to grow at a higher temperature than the majority of the isolated strains is not considered to be a distinguishing feature.

On the basis, also, of organic acid utilization Bact. ammoniagenes resembles more closely the saprophytic strains than the animal pathogens, C. renale and C. ovis. This is shown in Table 18 for representatives of the isolated strains and for

Table 17

Comparison of the characters of the urease-positive strains of Corynebacterium with those of named cultures

	Isolated strains of Corynebacterium	Bact. ammoniagenes	C. renale	C. ovis
Diastase production	7+ 6- or slight (9 not tested)	-	-	-
Litmus milk	16 no change; 1 reduction; 5 rennet clot, reduction	No change	Alkaline, slow digestion	No change
Gelatin liquefaction	+	-	-	-
Haemolysis on blood agar	-	-	-	+
Pigment on agar	White to cream	Pale cream	Pale cream	White
Growth at 37°	2+ 20-	+	+	+

Table 18

Organic acid utilization by urease-positive coryneform organisms

Representatives of the isolated strains	Utilization of: Acetate	Citrate	Succinate	Gluconate	Lactate	Propionate	Benzoate
7-11	+K	-	-	+	+K	-	-
7A-26	+K	+K	+K	+	+K	-	+
7-7	+K	+K	+K	+	+K	+K	-
7A-1	-	-	-	+	-	-	-
12-2	+K	+K	-	+	+K	+K	-
Bact. ammoniagenes	+K	+K	+K	+sl.K	+K	+K	-
C. renale	-	-	-	-	-	-	-
C. ovis	-	-	-	-	-	-	-

K = alkaline reaction; sl. = slight.

the named cultures.

Bact. ammoniagenes appears in Bergey's Manual (1948) in the genus Bacterium. Its transfer to the genus Brevibacterium is suggested by Breed (1953) but in view of its coryneform morphology it seems more reasonable to associate it with the diphtheroids.

The presence of strains of Bact. ammoniagenes in built-up poultry litter would result in the hydrolysis of urea, thus completing the breakdown of uric acid to ammonia.

Corynebacteria producing excess vitamin B12-active compounds

Strains capable of producing vitamin B12-active compounds were detected by their ability to support the growth of the Davis mutant of Escherichia coli in a vitamin B12 deficient-medium.

Twenty-eight positive strains were found representing 9% of the total number of corynebacteria examined. Only one of these is a uric acid decomposer placed in group C. The other 27 strains are similar to each other in morphology, occurring as angularly arranged rods, 1-1.2 x 2-3μ, in young culture. None is motile. Colonies on agar, except for pigment production, are also alike being usually 1 mm. or less in diameter, low convex, smooth, glistening, entire, opaque and butyrous. All grow well in glucose agar but produce no change in the pH. All are diastase-negative. Inorganic nitrogen is not utilized. In other characteristics the strains vary and these differences are shown

in Table 19. The one uric acid-decomposing strain is included for comparison; this strain also has no action on glucose or starch.

Type A strains are similar to Corynebacterium nubilum described by Jensen (1934). The other types B, C and D have not been identified with named species but they seem to resemble most closely the Corynebacterium liquefaciens group. Jensen's (1934) strains all produced slight acidity from glucose but generally had little action on other carbohydrates. Utilization of organic acids by types B, C and D shows a similar pattern as is shown for representative strains in Table 20. Types C and D were found to be capable of decomposing uracil and thymine forming ammonia and urea; in this respect they resemble the B12-positive uric acid decomposer except that this strain forms ammonia only, being urease-positive.

None of these characteristics provides any means by which the organisms can be distinguished from other corynebacteria that occur in poultry litter but show no vitamin B12 activity.

None of these strains of corynebacteria was isolated from the sample of unused litter or from the sample of poultry droppings. However each of the samples of built-up litter listed in Table 1 yielded one to four positive strains. No conclusions can be drawn from these small figures but it would appear that no one type of litter especially favours their development.

Table 19

Characters of the strains of Corynebacterium producing an excess of vitamin B12-active compounds

	A	B	C	D	Uric acid decomposer
No. of cultures	11	7	2	7	
Litmus milk	No change	No change	No change	Clot, redn., peptn.	Clot, redn., peptn.
Gelatin liquefaction	-	-	+	+	-
Pigment on agar	Pink orange-pink	None	None	None	Orange
Growth at 37°	1+ 10-	-	-	-	-

Table 20

Organic acid utilization by vitamin B12-producing strains of Corynebacterium

Representative strains of	Utilization of: Acetate	Citrate	Succinate	Gluconate	Lactate	Propionate	Benzoate
A	+K	-	-	-	-	-	-
B	+K	-	-	-	+K	+sl.K	+
C	+K	-	-	-	+K	+sl.K	+sl.K
D	+K	-	-	-	+K	+K	+sl.K
Uric acid decomposer	+K	+K	+K	+sl.K	+K	+K	-

K = alkaline reaction; sl. = slight.

Other corynebacteria isolated from poultry litter and droppings

Excluding the strains concerned in uric acid decomposition and vitamin B12 synthesis leaves 128 strains of corynebacteria for which no particular function has been found. This represents 41% of the total number examined.

These fall broadly into two groups, A and B, on the basis of cell and colony size. Group A strains occur in young culture as rods, 0.8-1.5 x 1.5-6μ; colonies on nutrient agar are 1-2 mm. in diameter; they grow well in glucose agar producing a slight to moderate acidity. Group B strains are smaller in size, produce small colonies (< 1 mm. in diameter) on nutrient agar and grow poorly in glucose agar. All strains of A and B fail to utilize either ammonia or nitrate as a nitrogen source. An examination of other characteristics in Table 21 shows that no sharp distinction exists between groups A and B.

Utilization of organic acids similarly fails to provide any means by which the strains can be satisfactorily divided into clearly defined types.

These strains agree most closely with Jensen's (1934) C. liquefaciens group being variable in their action on glucose and starch and showing no proteolytic activity in milk.

Nutritional requirements of the coryneform bacteria

When first examined it was found that Bact. globiforme, C. helvolum, C. ureafaciens and most of the uric acid-decomposing

Table 21

Characters of the strains of Corynebacterium for which no particular function has been found

	A	B
No. of cultures	86	42
Reaction in glucose agar	Slight to moderate acidity	Slight acidity or no change
Diastase production	13+ 16- 10 slight (only 39 tested)	7+ 6- 9 slight (only 22 tested)
Litmus milk	68 no change; 2 reduction; 16 rennet clot, reduction	No change
Gelatin liquefaction	71+ 15-	32+ 10-
Pigment on agar	Cream, pale yellow to yellow	Pale yellow or none
Growth at 37°	13+ 73-	6+ 36-
Motility	-	10+ 32-

strains of group B could grow in a glucose-mineral salts medium with either ammonia or nitrate as a source of nitrogen. However when tested again later variable results were obtained, some of the formerly positive organisms failing to grow when provided with only an inorganic source of nitrogen.

In the early work the glucose had been sterilized in the medium but in later work it was autoclaved separately and added to the medium aseptically. A comparison made between media containing glucose sterilized by these two methods failed to reveal any difference in the ability to support the growth of the organisms concerned.

It was decided to investigate further the effect of inoculum size on the ability to grow using inorganic nitrogen. For this purpose Bact. globiforme and C. helvolum were selected as test organisms. The following liquid media, tubed in 5 ml. quantities, were used:

(a) Glucose-NH_4^+.

(b) Glucose-NO_3^-.

(c) Citrate-NH_4^+.

(d) Peptone-yeast extract as a control.

From growth on peptone-yeast extract agar slopes, suspensions of the two test organisms were made in saline. Eleven succeeding dilutions were made by adding 2 ml. of the previous dilution to 2 ml. of saline and mixing. A volume of 0.1 ml. of each dilution was then added to each of the

media (a) to (d). The inoculated tubes were incubated at 22°, the final readings at 14 days being shown in Table 22.

As can be seen from the results, the ability to grow in media containing only an inorganic source of nitrogen depends on the number of cells inoculated. Small inocula fail to develop with inorganic nitrogen although the same number of cells produce heavy growth in peptone-yeast extract broth. Variation in the size of inoculum would therefore account for the variable results encountered in earlier work.

From these findings it is obvious that in any nutritional study of the corynebacteria, careful attention must be given to the size of inoculum used. Tests based on the ability of organisms to grow in simple media with no accessory growth factors become valueless unless the inoculum size is also specified.

During the present investigation the nutrition of the coryneform bacteria was not studied in detail. From the results of a few tests, however, it could be concluded that their requirements vary from simple to complex and provide no means for separating the strains into clearly defined types.

Systematic position of the coryneform bacteria isolated from poultry litter and droppings

The problem arises as to which genus these isolates from poultry litter and droppings should be assigned.

Since its creation by Lehmann & Neumann (1896) the

Table 22

The ability of Bact. globiforme and C. helvolum to utilize inorganic nitrogen

Diln.*	Bact. globiforme				C. helvolum			
	Glucose		Citrate	Peptone -yeast extract	Glucose		Citrate	Peptone -yeast extract
	NH_4^+	NO_3^-	NH_4^+		NH_4^+	NO_3^-	NH_4^+	
1	+++sl.A	+++	+++sl.K	+++	+++sl.A	+++	+++K	+++
2	+++sl.A	+++	+++sl.K	+++	++sl.A	++	++sl.K	+++
3	+++sl.A	+++	+++sl.K	+++	++	++	++sl.K	+++
4	++sl.A	++	++sl.K	+++	++	+	++	+++
5	++sl.A	++	++	+++	-	-	+/-	+++
6	++	++	+	+++	-	-	-	+++
7	+	+	+	+++	-	-	-	+++
8	+/-	-	-	+++	-	-	-	+++
9	-	-	-	+++	-	-	-	+++
10	-	-	-	+++	-	-	-	+++
11	-	-	-	+++	-	-	-	+++
12	-	-	-	+++	-	-	-	+++

Growth is assessed by the number of + signs; +++ is heavy growth
A = acidic reaction; K = alkaline reaction; sl. = slight.

* Progressive 2-fold dilutions were used.

genus Corynebacterium has come to include a great variety of organisms differing widely in morphology and physiological activity. Conn & Dimmick (1947) attempted to simplify the situation by removing the soil corynebacteria into a newly proposed genus Arthrobacter with Arthrobacter globiforme as the type species. One characteristic of this genus is the ability to use inorganic nitrogen (ammonia or nitrate) as a source of nitrogen.

As has been shown during the present investigation, the ability of some organisms to grow in media containing only an inorganic source of nitrogen depends on the size of inoculum used. To base a generic distinction on such a characteristic seems to be quite unsatisfactory. Furthermore Arthrobacter is intended to cater for all the soil corynebacteria many of which have been shown to require organic nitrogen (Jensen, 1934; Topping, 1936; Taylor, 1938; Lochhead & Chase, 1943).

Recently the tendency has been to place corynebacteria of soil origin in the genus Arthrobacter irrespective of their nutritional requirements. Thus Lochhead & Burton (1953) included in this genus Arthrobacter terregens, nutritionally an extremely fastidious organism. These authors likewise consider that coryneform organisms from soil, for which vitamin B12 is an essential nutrilite, belong in this genus (Lochhead & Burton, 1955). Sacks (1954) proposed the name Arthrobacter citreus for a yellow-pigmented isolate from

chicken faeces; it differed from Arthrobacter helvolum in its inability to hydrolyse starch and lack of growth on media with only an inorganic source of nitrogen.

Lochhead, in his proposed key for Arthrobacter in the forthcoming seventh edition of Bergey's Manual, has made provision for organisms which require organic nitrogen. He has, however, based the primary division of the genus on the ability to grow with inorganic nitrogen and citrate as the only energy source, Arthro. globiforme being positive in this respect. Such a division is unsatisfactory as Arthro. globiforme fails to grow under such conditions if the inoculum is sufficiently small.

If nutritionally exacting organisms are admitted to Arthrobacter, the distinction between this genus and Corynebacterium then becomes one of habitat. The difficulty of applying such a distinction in practice becomes obvious when one considers these isolates from poultry litter and droppings. Should they be associated with soil or the animal?

Bisset & Moore (1949) proposed the name Jensenia for the soil diphtheroids. These were distinguished by the unicellular nature of the individual bacilli, Nocardia and Corynebacterium being multicellular. Unfortunately these authors restricted their observations to a limited number of strains and failed to examine any of the soil diphtheroids

previously described by soil bacteriologists. Their type culture Jensenia canicruria (Bisset & Moore, 1950) is, moreover, considered to be a nocardia (Clark, 1952). Jensenia has therefore not been considered as a possible generic name for any of the organisms discussed here.

Because of the unsuitability of using Arthrobacter (either as originally defined or in its less restricted sense) or Jensenia as a generic name for the isolates from poultry litter, it seems best to follow Jensen's (1952) lead and leave these strains in the genus Corynebacterium until more is known of the soil diphtheroids as a whole.

Nocardia

Members of this genus were only occasionally detected among the predominant flora of built-up poultry litter. Four strains isolated are of interest.

Three of these decompose uric acid. They form a branching mycelium in very young culture; in other characteristics they cannot be distinguished from the uric acid-decomposing corynebacteria placed in group A.

These three strains have not been identified with any of the species of Nocardia listed in Bergey's Manual (1948). They resemble most closely two strains (designated A and M) from soil, described by Jensen (1934) as being intermediate in their characters between Proactinomyces (Nocardia) and Corynebacterium.

Because of their similarity to the uric acid-decomposing corynebacteria placed in group A, these three strains might best be regarded as belonging to the same species as Bacterium globiforme. This organism has been reported by Conn & Dimmick (1947) to show branching forms in certain liquid media.

One strain of Nocardia was found which forms an excess of vitamin B12-active compounds. This strain produces in young culture a fine branching Gram-positive mycelium which in older culture disintegrates into rod and coccal forms. It has no action on glucose and starch; gelatin is not liquefied.

Nitrate is reduced to nitrite. In litmus milk an alkaline reaction develops and in old cultures the milk is partially cleared. This description fits that given for Nocardia opaca in Bergey's Manual (1948). Phenol utilization was not determined as Jensen (1932) does not consider this to be an essential characteristic of the species.

Streptomyces

Four of the Streptomyces strains isolated decompose uric acid.

Colonies on nutrient agar are rough and dull, white, opaque and of a tenacious consistency. They smell characteristically of damp earth. On uric acid agar clearing slowly occurs; ammonium urate also appears although no appreciable urease activity is demonstrated in urea broth. All are strict aerobes. All strains show diastatic activity and one strain produces slight acidity in glucose agar. In litmus milk the dye is reduced; a rennet-like clot is formed and rapidly digested. Gelatin is actively liquefied.

These strains have not been definitely identified. They resemble Streptomyces albus except for their ability to hydrolyse starch. Jensen (1931), however, has described diastase-positive strains which he tentatively identified as this species.

Only one of these strains was isolated from built-up poultry litter; the other three strains originated from the sample of unused litter. In view of their infrequent occurrence, streptomyces are evidently not of major importance in the decomposition of uric acid in built-up poultry litter.

The three strains from unused litter are capable of synthesizing vitamin B12-active compounds. It is possible that streptomyces may contribute to the vitamin B12 content

of built-up litter even if present in small numbers.

Micrococcus

The 32 strains of *Micrococcus* studied were isolated from high dilution plates used for estimating the total count of poultry litter and droppings.

All the strains are aerobic, non-motile, catalase-positive, Gram-positive cocci. The size of the cells varies a little among strains from 1-1.5μ in diameter. The cells are commonly arranged in pairs and irregular groups but sometimes tetrads are formed. This latter feature, however, is not definite enough to warrant the separation of the strains concerned into the genus *Sarcina* and all have been placed in the genus *Micrococcus*.

None of the strains has any action on uric acid. All failed to produce detectable amounts of vitamin B12-active substances.

The strains have been grouped according to the characters shown in Table 23.

Group A

The 26 strains placed in this group would be placed by Shaw *et al*. (1951) in their subgroup 3 (*Staphylococcus lactis*). They are all coagulase-negative, ferment glucose but do not form acetoin, and do not produce a pink pigment.

Groups A1 and 2 agree with the description given in Bergey's Manual (1948) for *Micrococcus pyogenes* var. *albus*. They have been separated here on the basis of urease

Table 23

Characters of the strains of Micrococcus isolated from poultry litter and droppings

	A1	A2	A3	A4	A5	B
No. of cultures	9	8	4	4	1	6
Fermentation of						
glucose	+	+	+	+	+	-
mannitol	+	+	+	+	-	-
Utilization of N compounds						
$NH_4H_2PO_4$	-	-	-	-	-	-
urea	-	-	-	-	-	-
Production of acetoin	-	-	-	-	-	-
Nitrite from nitrate	+	+	+	-	+	-
Urea hydrolysis	+	-	+ or -	+	+	-
Litmus milk	Acid, clot, redn.	Acid, slight clot	Acid	Acid, clot, redn.	Acid, clot, redn.	No change
Gelatin liquefaction	+	+	-	+	+	-
Pigment on agar	White	White	White to yellow	Yellow	White	None

production. This species has been isolated from the intestinal contents of poultry by Menes & Rochlin (1929) and from the caecal contents of turkeys by Harrison & Hansen (1950a).

Group A3 strains have been identified as Micrococcus aurantiacus; three of the four strains are urease-positive. Groups A4 and 5 have been identified as Micrococcus flavus and Micrococcus epidermidis respectively. These three species were also reported by Harrison & Hansen (1950a) as being present in the caecal contents of turkeys.

The urea-decomposing strains differ from Micrococcus ureae in their inability to utilize urea as the only nitrogen source. This species also produces a slightly alkaline reaction in litmus milk.

Group B

These 6 strains belong in subgroup 5 (Staphylococcus afermentans) of Shaw et al. (1951). They are coagulase-negative and fail to ferment glucose. No growth occurs at 37°. Physiologically they are very inactive. It has not been possible to identify these strains with any named species in Bergey's Manual (1948).

Approximately half the strains of Micrococcus examined were found to produce the enzyme urease. In poultry litter these strains would decompose urea with the formation of ammonia.

Pseudomonas

Twenty-nine strains belonging to the genus Pseudomonas were isolated.

All are motile Gram-negative rods which produce a green diffusible fluorescent pigment. None grow at 37°. On nutrient agar colonies are large, low convex, smooth and glistening, translucent to opaque, and varying from colourless to pale yellow. In a glucose agar stab culture growth is aerobic; a slight acid reaction at the surface reverts after a few days to slight alkalinity. Starch is not hydrolysed. Strains which decompose uric acid form urea as an end-product; all strains are urease-negative. Other characteristics of the strains are given in Table 24.

Group A strains agree with the description of Pseudomonas ovalis Chester; four strains differ slightly in that a rennet clot is produced in litmus milk after 3-4 weeks' incubation at 22°. Two representative strains from this group were found to decompose creatine and creatinine in a medium containing mineral salts and 0.05% yeast extract. Ammonia and urea were detected as end-products of the reaction. Nimmo-Smith & Appleyard (1956) have identified as Ps. ovalis a pseudomonad, isolated from ground used as a chicken-run, which forms ammonia and urea in the decomposition of creatine but which has no action on creatinine. It is probable that the strains isolated from poultry litter and droppings and identified as Ps. ovalis

Table 24

Characters of the strains of Pseudomonas isolated from poultry litter and droppings

	A	B
No. of cultures	14	15
Litmus milk	At first slightly alkaline, after 14 days becoming slightly acid (4 late rennet clot)	Rennet clot later digested, litmus reduced
Gelatin	No liquefaction or only slight	Strong liquefaction
Nitrate peptone water	No action	12 no action, 2 form nitrite, 1 forms gas
Uric acid decomposition	+	14+ 1-
Vitamin B12 synthesis	+	12+ 2- (1 not tested)

are similar to the uric acid decomposers identified as Bacillus fluorescens non-liquefaciens by Liebert (1909).

Liebert (1909) also stated that uric acid was decomposed by Bacterium calco-aceticum but gave no description of this organism. The organism referred to by Beijerinck (1911) as Micrococcus calco-aceticus was undoubtedly identical with Bact. calco-aceticum as shown by the index of organisms in the 6th part of his collected papers (Beijerinck, 1940). This same organism was later renamed Pseudomonas calco-acetica by Clifton (1937). Unfortunately neither Beijerinck nor Clifton contributed sufficient information about this organism for it to be recognized and it has not been possible to compare it with the strains isolated during the present investigation.

No specific names have been given to the group B strains; it is possible, however, that the strains which decompose uric acid are similar to those isolated and identified as Bacillus fluorescens liquefaciens by Liebert (1909).

The one group B strain which forms gas from nitrate is capable of growing anaerobically in the presence of nitrate. It decomposes uric acid. It fails to grow at 37° and thus differs from Pseudomonas aeruginosa, a characteristic feature of which is the ability to grow at 41° (Haynes, 1951). The strain did not show, on isolation, the colony shape and tenacious consistency characteristic of Pseudomonas stutzeri (van Neil & Allen, 1952); this species, moreover, fails to liquefy gelatin,

is non-fluorescent and grows well at 37° in contrast with the isolated strain. This strain therefore differs from Liebert's (1909) denitrifiers which were capable of decomposing uric acid; these he identified as Bacillus pyocyaneus (Ps. aeruginosa) and Bacillus stutzeri (Ps. stutzeri).

The majority of the pseudomonads decompose uric acid. Although they were not found among the predominant flora except in the sample of unused litter, they undoubtedly assist in the decomposition of uric acid in built-up litter. They probably also contribute to its vitamin B12 content as most produce an excess of this compound in artificial culture.

Alcaligenes - Achromobacter

Alcaligenes

The generic name Alcaligenes is used for the Gram-negative isolates from poultry litter and droppings which produce an alkaline reaction in litmus milk and form no acid from sugars. All the strains isolated proved to be of interest. They originated from the majority of the built-up litter samples and from the sample of poultry droppings but not from the sample of unused litter.

All strains appear as Gram-negative cocco-bacilli or short rods, 0.5-1 x 1-2μ, arranged singly, in pairs and occasionally in short chains. The interiors of the cells often fail to stain. Some of the motile strains show typical peritrichous flagellation. The remainder of the motile strains show only a few cells with bipolar or peritrichous flagella; other cells possess only a single flagellum in either a polar or lateral position. Colonies on agar are circular, 0.5-2 mm. in diameter, low convex, smooth and glistening, entire or slightly undulate, translucent and often viscous. Some of the motile strains produce a pale yellow pigment. Growth at 37^{o} is variable. All are strict aerobes except the denitrifiers which are able to grow anaerobically in the presence of nitrate.

All strains fail to liquefy gelatin and have no action on starch. They fail to produce acidity in glucose peptone

water with brom-thymol blue as indicator. Strains were also inoculated into a similar medium with the glucose concentration raised from 0.5 to 1.0% and the peptone concentration lowered from 1.0 to 0.2%. Growth in this latter medium was generally poorer and also failed to demonstrate acid production. Many of the strains produce a viscous growth in litmus milk and with some the milk becomes translucent after long incubation; these characteristics were not definite enough to warrant separation of the strains concerned.

Other characteristics of the organisms are shown in Table 25. It is evident that none of the tests employed can be used satisfactorily to divide this group of organisms. The strains producing significant amounts of vitamin B12-active compounds cannot be distinguished, on the basis of these characteristics, from the other strains.

Leifson & Hugh (1954) have recently proposed the name Alcaligenes denitrificans for strains of this genus able to produce gas from nitrate. These were described as motile Gram-negative rods which have no action on glucose and produce an alkaline reaction in litmus milk. They fail to liquefy gelatin and are urease-negative.

Thirteen of the strains isolated from poultry litter and droppings produce gas from nitrate. Two of these conform to the above description, 5 strains differ in being able to hydrolyse urea and 6 others are non-motile. These 13 strains,

Table 25

Distribution of characters in strains of Alcaligenes isolated from built-up poultry litter and droppings

		Uric acid decomp.		Urea hydrolysis		Nitrate redn.		Vit. B12 prodn.	
No. of cultures	46	+	-	+	-	+	-	+	-
Motility	+31	25	6	13	18	11	20	7	24
	-15	12	3	3	12	8	7	3	12
Uric acid decomposition	+37	..	..	8	29	11	26	9	28
	- 9	..	..	8	1	8	1	1	8
Urea hydrolysis	+16	..	..	..	..	7	9	2	14
	-30	..	..	..	..	12	18	8	22
Nitrate reduction	+19	..	..	..	..	..	..	4	15
	-27	..	..	..	..	..	..	6	21
Vit. B12 production	+10	..	..	..	..	..	..	..	..
	-36	..	..	..	..	..	..	..	..

however, show the same variation in other characteristics as do the strains which reduce nitrate to nitrite and also those which have no action on nitrate (Table 26). To separate the denitrifiers on the basis of this one characteristic does not, therefore, appear to be logical and all the strains are regarded as comprising one group.

The failure to liquefy gelatin and the ability of many of the strains to produce a viscous type of growth in litmus milk suggest that this group of 46 strains is similar to Alcaligenes viscosus. This species is reported in Bergey's Manual (1948) as being variable in motility.

Achromobacter

Gram-negative organisms which do not show an alkaline reaction in litmus milk and which do not produce a distinct yellow pigment have been placed in the genus Achromobacter. A large number of such organisms were isolated from poultry litter and droppings. Some of these were of particular interest either in connection with the decomposition of uric acid to ammonia or in the synthesis of vitamin B12. These were examined in greater detail.

The 49 strains examined originated either from the sample of poultry droppings or from 8 of the built-up litter samples, 7 of which had a pH less than 8. No strains were detected either in the sample of unused litter or in 5 built-up litter samples having a pH greater than 8. There is some indication

Table 26

Action on nitrate by strains of Alcaligenes isolated from built-up poultry litter and droppings

	No action on nitrate		Nitrate reduced to nitrite		Gas produced from nitrate	
No. of cultures	27		6		13	
Motility	20+	7-	4+	2-	7+	6-
Uric acid decomposition	26+	1-	3+	3-	8+	5-
Urea hydrolysis	9+	18-	2+	4-	5+	8-
Vit. B12 production	6+	21-	4+	2-	-	

therefore, that these organisms are not among the predominant Gram-negative flora on unused litter or in strongly alkaline built-up litter.

Morphologically they resemble the strains of Alcaligenes which have previously been described; motile strains show the same variation in flagellation. Growth on agar is also similar but never becomes viscous; pigmentation varies from none to pale yellow. Growth at 37° is variable. All are strict aerobes except for one denitrifier which can grow anaerobically with nitrate.

All strains fail to liquefy gelatin. Growth in litmus milk does not become viscous; the reaction is neutral to slightly acidic. Table 27 shows the distribution of some of the characteristics of the strains. Action on glucose was determined in glucose peptone water containing brom-thymol blue. Not all the strains producing acid in this medium showed an acidic reaction in litmus milk.

As with the strains of Alcaligenes none of the tests employed can be used satisfactorily to divide this group. To separate these strains on the basis of reaction in litmus milk and action on nitrate, as in Bergey's Manual (1948), does not seem to be reasonable and for the time being no specific names have been assigned to them.

The activity of these organisms in built-up poultry litter is possibly restricted to material which has not become

Table 27

Distribution of characters in strains of Achromobacter isolated from built-up poultry litter and droppings

		Acid from glucose		Acid in lit.milk		Uric acid decomp.		Urea hydrolysis		Nitrate redn.		Vit. B12 prodn.	
No. of cultures	49	+	-	+	-	+	-	+	-	+	-	+	-
Motility	+14	1	13	0	14	13	1	1	13	2	12	6	8
	-35	28	7	26	9	5	30	20	15	4	31	10	25
Acid from glucose	+29	..	..	26	3	5	24	20	9	4	25	4	25
	-20	..	..	0	20	13	7	1	19	2	18	12	8
Acid in litmus milk	+26	..	..	..	..	5	21	20	6	5	21	1	25
	-23	..	..	..	..	13	10	1	22	1	22	15	8
Uric acid decomposition	+18	..	..	..	..	..	..	1	17	6	12	5	13
	-31	..	..	..	..	..	..	20	11	0	31	11	20
Urea hydrolysis	+21	..	..	..	..	..	..	..	..	0	21	1	20
	-28	..	..	..	..	..	..	..	..	6	22	15	13
Nitrate reduction	+ 6	..	..	..	..	..	..	..	..	..	..	0	6
	-43	..	..	..	..	..	..	..	..	..	..	16	27
Vit. B12 production	+16	..	..	..	..	..	..	..	..	..	..	..	..
	-33	..	..	..	..	..	..	..	..	..	..	..	..

excessively alkaline. In built-up litter of pH greater than 8, they appear to be replaced by strains of Alcaligenes. Both groups are concerned in the decomposition of uric acid and in the synthesis of vitamin B12.

Relationship between strains of Alcaligenes and Achromobacter

From the preceding work it can be seen that a close relationship exists between strains placed in Alcaligenes and those in Achromobacter. Further tests, carried out on representative strains from each genus, substantiated this. The ability to grow in simple media and the utilization of various organic acids could not be used for differentiating the two genera as is shown in Table 28. Thus although the strains have been placed in two genera on the basis of reaction in litmus milk, in other characteristics such a clear-cut distinction does not exist.

Table 28

Further characteristics of representative strains of Alcaligenes and Achromobacter

	Alcaligenes					Achromobacter				
Strain	a	b	c	d	e	f	g	h	i	j
Ability to grow in:										
Glucose-NH_4^+ medium	-	-	-	-	-	-	-	-	-	-
Citrate-NH_4^+ medium	-	+	-	-	+	+	-	-	-	-
Utilization of organic acids:										
Acetate	+K	+K	+K	+K	+K	+K	+K	+K	+K	+K
Citrate	+K	+K	+K	+K	+K	+K	-	-	-	-
Succinate	-	+K	+K	+K	+K	+K	+K	-	-	+sl.K
Gluconate	-	-	+	+sl.K	+	+	-	-	-	-
Lactate	+K	+K	+K	+K	+K	+K	+K	-	-	+sl.K
Propionate	-	+K	+K	+K	-	+K	+K	-	-	-
Benzoate	-	-	-	-	-	-	-	-	-	-
Oxalate	+K	-	-	-	-	-	-	-	-	-

K = alkaline reaction; sl. = slight.

Flavobacterium

Five strains of Flavobacterium which were found to be active synthesizers of substances having vitamin B12 activity were examined in greater detail. These show no action on uric acid or urea.

Four of these originated from one sample of built-up poultry litter. They appear as Gram-negative cocco-bacilli 0.5 x 1-1.5 and are non-motile. Growth on nutrient agar was poor but good growth was obtained on peptone-yeast extract agar with the production of a bright yellow pigment. Colonies are circular, convex, smooth and glistening, translucent to opaque and are of a butyrous consistency. Growth occurs at 37°. These strains can use ammonia as a source of nitrogen in a glucose mineral salts medium; slight acidity is produced. Starch is not hydrolysed. Nitrate is not reduced. Gelatin is not liquefied and litmus milk remains unchanged. On the basis of these characteristics the strains have been identified as Flavobacterium solare.

The remaining strain placed in the genus Flavobacterium originated from the sample of unused litter. This is a Gram-negative rod, 1 x 2-4μ, motile by means of peritrichous flagella and producing yellow growth on agar media. It shows no growth at 37°. Slight acidity is produced in glucose agar; starch is not hydrolysed. Nitrate is not reduced. Gelatin is liquefied. In litmus milk the indicator becomes

reduced and a rennet clot is formed. It has not been possible to identify this organism with any of the named species listed in Bergey's Manual (1948).

Cytophaga

Eight strains of Cytophaga were isolated from the sample of poultry droppings and from two samples of built-up litter.

They are small Gram-negative cocco-bacilli, approximately 0.5 x 0.5-1μ. Colonies on agar are yellow, translucent and iridescent. The centres of the colonies are raised whereas the peripheries are thin and spreading. In hanging drops no obvious motility was observed but some of the cells showed flexing movements. On the basis of these characteristics the eight strains have been placed in the genus Cytophaga.

All produce a slightly alkaline reaction in glucose agar; starch is not attacked. Gelatin is actively liquefied. Nitrate is not reduced. One strain produces no change in litmus milk, the remaining strains bring about coagulation with reduction of the litmus. Uric acid is not attacked and vitamin B12-active substances were not detected in cultures of these strains.

The outstanding feature of these strains is their ability to hydrolyse urea actively. This is evident from the titration figures which varied from 4.1 to 5.6 ml.

The cytophagas have been given particular attention in the past in connection with their ability to decompose cellulose. It is apparent from this work that they are also concerned in other decomposition processes in nature. Even though present in small numbers in built-up litter they probably assist in the decomposition of urea to ammonia.

SUMMARY

A study has been made of organisms isolated from poultry litter and droppings. Many of these are concerned in the decomposition of uric acid and in the synthesis of compounds having vitamin B12 activity. The following genera were found to be represented.

Corynebacterium

Approximately 43% of the isolates studied are capable of decomposing uric acid. These strains do not fall into distinct types but present a continuous spectrum in their characteristics. Bacterium globiforme, Corynebacterium helvolum, Bacterium linens, Corynebacterium ureafaciens and Topping's strains 81 and 178 were all found to be uric acid decomposers and could be related to the strains isolated from poultry litter and droppings.

Bacterium ammoniagenes, a coryneform organism capable of hydrolysing urea, was found to be similar to the urea decomposers from poultry litter and droppings.

Other strains studied included those which produce excess amounts of vitamin B12-active compounds and others for which no particular function was found.

The use of the genus Arthrobacter for the soil corynebacteria is thought to be unsatisfactory. Even if nutritionally exacting organisms are admitted to this genus its distinction from Corynebacterium becomes one of habitat,

a characteristic which is difficult to apply in practice. It is suggested that the coryneform bacteria isolated from poultry litter and droppings should, for the present, be recognized as members of the genus Corynebacterium.

Nocardia and Streptomyces

A few organisms from these genera were isolated.

Micrococcus

The majority of micrococci isolated were identified as Micrococcus pyogenes var. albus. Smaller numbers of Micrococcus aurantiacus, Micrococcus flavus and Micrococcus epidermidis were encountered.

Pseudomonas

Approximately half the pseudomonads isolated were identified as Pseudomonas ovalis; the remainder were not identified with named species.

Alcaligenes - Achromobacter

Organisms placed in these two genera were shown to be closely related. Those placed in the former genus are similar to Alcaligenes viscosus.

Flavobacterium

A few strains were placed in this genus; some of these were identified as Flavobacterium solare.

Cytophaga

The cytophagas isolated differ from previously named species; these strains actively hydrolyse urea.

PART IV

THE MICROBIOLOGICAL ASSAY OF VITAMIN B12; THE OCCURRENCE OF THIS VITAMIN IN BUILT-UP POULTRY LITTER AND ITS PRODUCTION BY MICRO-ORGANISMS ISOLATED FROM THIS SOURCE

THE MICROBIOLOGICAL ASSAY OF VITAMIN B12; THE OCCURRENCE OF THIS VITAMIN IN BUILT-UP POULTRY LITTER AND ITS PRODUCTION BY MICRO-ORGANISMS ISOLATED FROM THIS SOURCE

INTRODUCTION

Originally proteins were regarded simply as a source of dietary nitrogen but with the recognition of the rôle of amino acids in nutrition the quality of a protein began to be considered in terms of its amino acid content. Vegetable protein had long been known to be inferior to animal protein in nutritional value and workers looked for a possible explanation in terms of amino acid deficiency. It was found that animal protein generally contained a more satisfactory mixture of amino acids than did vegetable protein and it was concluded that it should be possible to compensate for the inferiority of vegetable protein by the addition of the essential amino acids. However it was still found that non-ruminant animals grew poorly when fed on a diet of vegetable origin even when the amino acid content appeared adequate.

The special value of animal proteins is now known to be due to their content of the so-called "animal protein factor". In the case of poultry, omission of this factor from the diet leads to low hatchability of eggs laid by the breeding hen and depressed growth of the chick. The early literature on this

aspect of poultry nutrition has been well reviewed by Zucker & Zucker (1950) and Smith (1950-1).

Other materials besides protein of animal origin were found to possess animal protein activity. Hammond (1944), Rubin & Bird (1946) and Whitson, Titus & Bird (1946) described the growth promoting effect of cow manure for chickens fed on a vegetable protein diet. A similar factor was reported in the faeces of hens (Rubin, Bird & Rothchild, 1946) the activity of which could be increased by incubation at 30° (McGinnis, Stevens & Groves, 1947). Groschke, Rubin & Bird (1948) found that seasonal variation in hatchability was associated with coprophagy, conditions being more favourable for synthesis of the factor in the voided faeces during the warmer months. Even soil was shown to possess slight activity (Stephenson, McGinnis, Graham & Carver, 1948). At this time the use of the built-up litter system of poultry-keeping was becoming popular in America. Experiments carried out at Ohio showed that built-up litter acted as a source of dietary factors essential for the growth of chickens and the hatchability of eggs (Kennard & Chamberlin, 1948; Kennard, Bethke & Chamberlin, 1948). Autoclaving built-up litter made it even more effective in promoting the growth of chicks on an all-vegetable diet (Halbrook, Winter & Sutton, 1950).

In 1948 came the almost simultaneous announcements by Rickes, Brink, Koniuszy, Wood & Folkers (1948) in America and

Smith (1948) in Britain of the isolation of the anti-pernicious anaemia factor, vitamin B12. The newly discovered compound also displayed strong animal protein activity for chicks and rats so that at first it was considered to be the animal protein factor itself. Evidence now accumulating suggests that there also exist other as yet unidentified factors of the complex but that vitamin B12 is responsible for most of the activity. In the following work therefore only this vitamin has been considered.

The fact that the animal protein activity of hen faeces increases on incubation suggests a microbial synthesis of the factor after voiding. One would expect then an increase in activity in built-up poultry litter where the droppings are allowed to accumulate in the litter material. That microbial synthesis is indeed possible is supported by the isolation from hen faeces (Stokstad, Page, Pierce, Franklin, Jukes, Heinle, Epstein & Welch, 1948) and built-up poultry litter (Halbrook, Cords, Winter & Sutton, 1950; Burton & Lochhead, 1951) of bacteria capable of producing animal protein or vitamin B12 activity.

It was the object of this section of the investigation to find out the extent to which vitamin B12 occurs in built-up poultry litter and to study the possible rôle of micro-organisms in its synthesis.

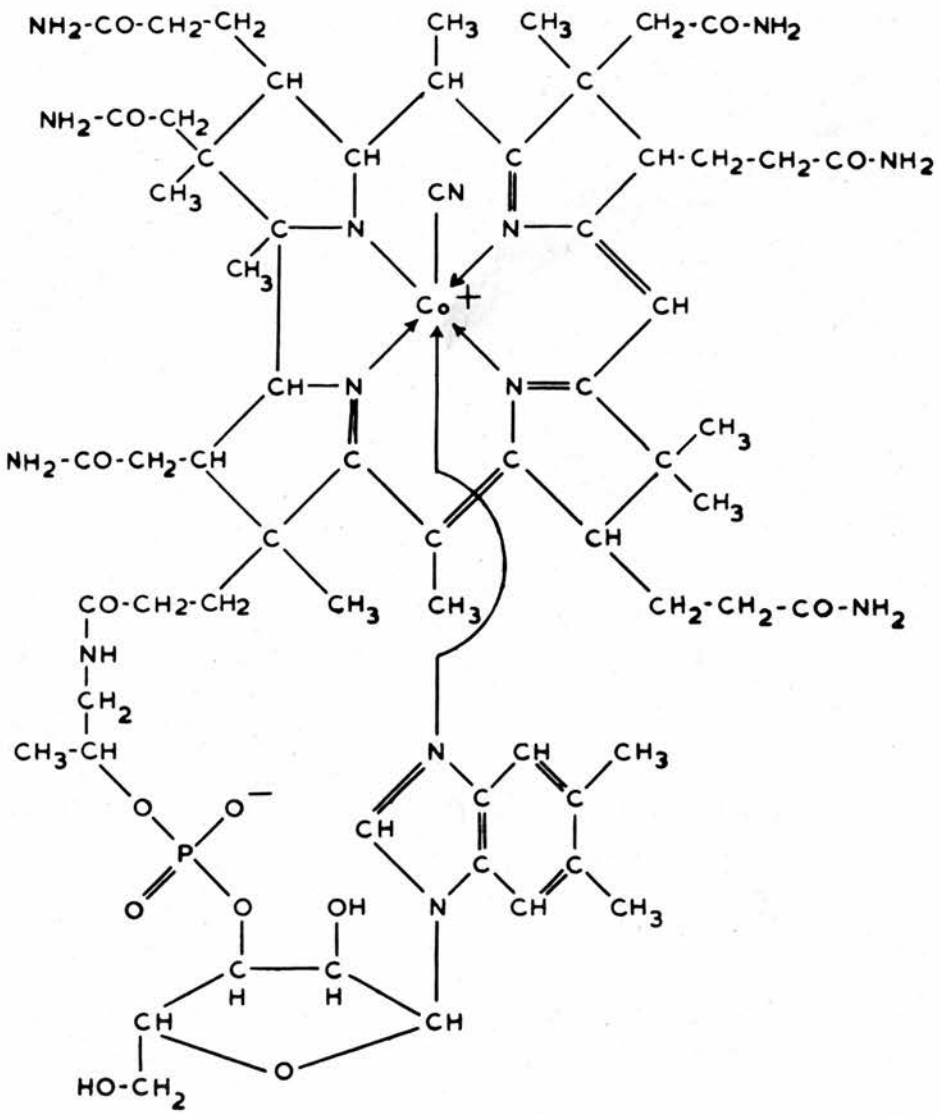
Vitamin B12 and its related compounds

For an appreciation of the difficulties involved in the assay of vitamin B12 in natural materials it is necessary to have some knowledge of the structure of vitamin B12 and its related compounds.

The complete structure for vitamin B12, put forward tentatively by Hodgkin, Pickworth, Robertson, Trueblood, Prosen & White (1955) and Bonnett, Cannon, Johnson, Sutherland, Todd & Smith (1955) is shown in Figure 1. Further evidence for this structure was provided, a little later, by Hodgkin, Kamper, Mackay, Pickworth, Trueblood & White (1956). The molecule contains a cyan group attached to the central cobalt atom. Replacement of this group by other anions gives a series of vitamin B12-like compounds, the so-called cobalamins. They are named according to the anion so that vitamin B12 itself is known as cyanocobalamin, the hydroxyl substituted compound as hydroxocobalamin (also known as vitamin B12a or B12b) and a nitrite substituted compound as nitritocobalamin (also known as vitamin B12c). In addition other more complex substitution compounds can be formed. The cobalamins vary in their activity for different assay organisms but as the common ones are easily reconverted by treatment with cyanide ions in a weakly acid solution to vitamin B12 itself (Wijmenga, Veer & Lens, 1950), they present no great problem in their assay.

FIGURE 1

STRUCTURAL FORMULA FOR VITAMIN B_{12}



There also occur in natural materials subjected to bacterial decomposition, such as rumen contents and faeces, other compounds which are not cobalamins but which are related to vitamin B12. The most common of these are factors A, B and C and also pseudovitamin B12 (Ford, Holdsworth, Kon & Porter, 1953) but small quantities of related compounds, factors D, E, F, G, H and I have also been isolated (Brown, Cain, Gant, Parker & Smith, 1955). These compounds show vitamin B12 activity to a varying degree for the commonly used assay organisms but of those so far isolated in the pure state only factor I (identical with vitamin B12III from sewage sludge) has displayed any activity towards higher animals (Brown et al., 1955).

The structure of some of these factors has been elucidated and it has been shown that they differ in the nature of the nucleotide portion of the molecule. Complete removal of the nucleotide from cyanocobalamin yields factor B. In other factors the difference has been found to lie in the nature of the base of the nucleotide. Where known these are shown in Figure 2. Obviously these compounds cannot be converted to vitamin B12 by treatment with cyanide as can the cobalamins.

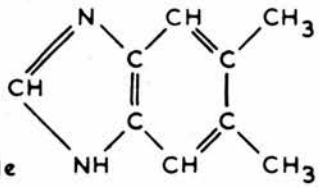
This outline gives a rough idea of the variety of compounds likely to be encountered when one is attempting to estimate the vitamin B12 content of natural materials. As was stated earlier the main compounds showing vitamin B12

FIGURE 2

STRUCTURE OF VITAMIN B_{12}-LIKE FACTORS

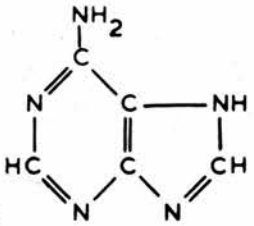
BASE OF NUCLEOTIDE

FACTOR



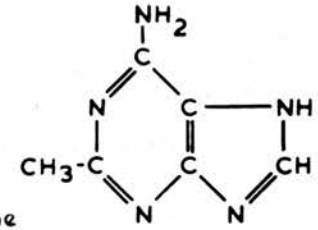
5:6-Dimethylbenziminazole

VITAMIN B_{12}



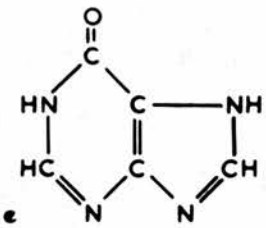
Adenine

PSEUDOVITAMIN B_{12}



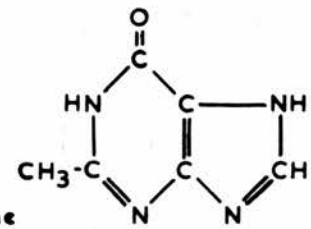
2-Methyladenine

FACTOR A



Hypoxanthine

FACTOR G



2-Methylhypoxanthine

FACTOR H

activity in faecal material are factors A, B and C, pseudovitamin B12 and vitamin B12 itself. Ford et al. (1953) were able to show that compounds previously reported as having vitamin B12 activity, such as vitamin B12m, pseudovitamin B12b, vitamin B12f and factor WR, could be resolved into varying proportions of the above constituents.

Except for factor D which is microbiologically inactive, factors A-I, pseudovitamin B12 and vitamin B12 all show activity for Escherichia coli and most show activity for Lactobacillus leichmannii (Ford & Porter, 1953; Brown et al., 1955). As only vitamin B12 and factor I (vitamin B12III) have any activity for higher animals, assays of natural materials using either E. coli or L. leichmannii cannot be taken as a measure of their vitamin B12 activity for animals. Ford (1953a) overcame this difficulty by employing a protozoan Ochromonas malhamensis for assay purposes. This organism was shown to be almost specific in its response to vitamin B12. O. malhamensis does respond to certain analogues of vitamin B12 prepared artificially (Ford, Holdsworth & Kon, 1955) but so far these have not been reported in nature and the assay results obtained for natural materials using this organism can be taken as a measure of vitamin B12 itself. This then is a much more useful assay organism when assessing the value of natural materials in the nutrition of animals.

EXPERIMENTAL AND RESULTS

Preparation of extracts for microbiological assay

The extraction of vitamin B12 from liver usually involves at some stage a proteolytic process. Wijmenga et al. (1950) showed that cyanide at pH 5.5 could replace this proteolysis by apparently removing a protein or peptide group originally linked to the vitamin B12 molecule. Ford (1952) confirmed that cyanide treatment of natural materials facilitated extraction. It also has the added advantage that cobalamins and related compounds are converted to their corresponding cyano-compounds.

The Shinfield workers (Ford, 1953b; Ford & Porter 1953) adopted the following procedure for the extraction of vitamin B12 from gut contents, faeces and other natural materials. An aqueous extract was steamed for 30 min. at pH 5.0 in the presence of cyanide, filtered or centrifuged and diluted as appropriate for assay. This extraction method was found to be inadequate for sow's milk and human milk which required papain digestion for releasing bound forms of the vitamin (Gregory, 1954).

The effect of cyanide and heat treatment on the extraction of vitamin B12 from poultry litter

The following experiment was carried out to ascertain the effect of cyanide and degree of heating on the extraction of vitamin B12-like compounds from poultry litter.

A 1 in 10 suspension of poultry litter was made in water and macerated for one minute. This was adjusted to pH 7.0 and six 20 ml. quantities measured into vials. To two of these was added 0.01% of potassium cyanide. Half the samples were autoclaved at 15 lb. for 15 min., the other half at 22½ lb. momentarily. The samples were then centrifuged and the supernatants tested for vitamin B12 activity on plates seeded with Escherichia coli (for details see assay method on p.104). To half of the samples to which no cyanide had been added during the heating treatment, 0.01% potassium cyanide was added just prior to testing. After incubation of the plates at 37° overnight the zones of response were measured and the results for each series of replicates averaged. The experimental details and results are given in Table 29.

Comparing the results it can be seen that the addition of cyanide before the heat treatment gives the highest vitamin B12 activity. The addition of cyanide just prior to testing (treatment C) increases the activity above that of treatment B but does not reach that of treatment A. For best results therefore cyanide should be added before the heat treatment. Coates, Ford, Harrison, Kon & Porter (1953) came to the same conclusion with calf rumen contents and faeces.

Autoclaving at 22½ lb. momentarily gave slightly higher activities than at 15 lb. for 15 min. when carried out in

Table 29

The effect of cyanide and heat treatment on the extraction of vitamin B12 from poultry house litter as determined on Escherichia coli seeded plates

Cyanide treatment	Average zone size in mm.	Heat treatment 15 lb./ 15 min.	Heat treatment 22½ lb./ moment.
A. 0.01% KCN added before heat treatment	20.0	X	
	20.0		X
B. No. KCN added	18.0	X	
	18.2		X
C. No KCN added before heat treatment but 0.01% added prior to testing	18.5	X	
	18.8		X

X = heat treatment used.

the absence of cyanide. In the presence of cyanide there was no such difference and the latter heat treatment was used for all subsequent extractions.

In this experiment the pH was adjusted to 7.0. Wijmenga et al. (1950) have stated that treatment with cyanide in a weakly acid solution readily converts cobalamins to cyanocobalamin. In later work, therefore, extracts were adjusted to pH 5.0 before autoclaving.

Methods of extraction adopted for poultry litter and cultures of micro-organisms

(a) Extraction of litter samples

The litter material was well mixed and a representative sample of 30 g. weighed out. An aqueous 1 in 10 suspension of this material was macerated for one minute which broke up lumps and gave a uniform mixture. This was adjusted to pH 5.0 with N-H_2SO_4 and 0.01% potassium cyanide added. Autoclaving of this mixture, at 15 lb. for 15 min., was carried out in a screw-capped bottle to prevent the escape of cyanide. After cooling most of the solid material was removed by centrifuging for 30 min. and the supernatant was sterilized by Seitz filtration.

(b) Extraction of cultures of micro-organisms

For the extraction of cultures of micro-organisms, 1% potassium cyanide was added to give a final concentration of 0.01% and the pH was adjusted with 0.1N-H_2SO_4 to approximately

4.8-5.0 using methyl red (concn. 0.0008%) as an internal indicator. The cultures were then autoclaved at 15 lb. for 10 min.

The effect of papain digestion on the extraction of poultry litter and cultures of micro-organisms

As vitamin B12 can exist in some natural materials in a bound form and is not released without a proteolytic treatment, it was advisable to verify that the extraction methods given above were adequate. For this purpose papain digestion was used.

(a) Extraction of poultry litter

As before, a 1 in 10 aqueous suspension was prepared, cyanide added and the pH adjusted to 5.0. To 100 ml. was added 20 ml. of water and to another 100 ml. portion was added 5 g. of papain in 20 ml. of water. Both were held in a water-bath at 63° for 1 hr. At the end of this digestion period both were autoclaved at 15 lb. for 15 min., allowed to cool, centrifuged and the supernatants resterilized at 22½ lb. momentarily.

(b) Extraction of cultures of micro-organisms

Cultures of four different organisms were tested:

(1) Strain 13-26 (nocardia).

(2) Strain 11-12 (corynebacterium).

(3) Strain 11-11 (micrococcus).

(4) Strain 11-14 (micrococcus).

Strains 13-26 and 11-12 had previously been shown to synthesize vitamin B12 in culture, strains 11-11 and 11-14 had failed to do so. When tested no proteolytic treatment had been used in the extraction. Strains 13-26 and 11-11 were non-proteolytic towards gelatin whereas strains 11-12 and 11-14 showed strong proteolysis. These were included to test the possibility of proteolytic enzymes produced by the organism itself having an effect on extraction.

Each of the four strains was inoculated into 30 ml. of a vitamin B12-deficient medium contained in a 100 ml. Erlenmeyer flask and incubated at 22° for 7 days. The cultures were then adjusted to pH 5.0 after addition of 0.01% of potassium cyanide. From each flask was measured out two 10 ml. quantities of the culture. To one of each pair was added 2 ml. of water and to the other 2 ml. of water containing 0.5 g. of papain. All eight tubes were held in a water-bath at 63° for 1 hr. They were then autoclaved at 15 lb. for 10 min., centrifuged and the supernatants resterilized at 22½ lb. momentarily.

(c) Vitamin B12 content of extracts

The two extracts of litter and the eight extracted cultures were assayed for their content of vitamin B12 using Ochromonas malhamensis as the test organism (for details of assay see p. 109). The results obtained are shown in Table 30.

From these results it would appear that there is no

Table 30

The effect of papain digestion on the extraction of poultry house litter and cultures of micro-organisms

Sample	Vitamin B12 content of extract mμg./ml. No papain digestion	Digestion with papain
Extract of litter	18.1	10.8
Extracts of cultures		
Strain 13-26	7.6	5.0
Strain 11-12	20.1	16.1
Strain 11-11)	No detectable activity	No detectable activity
Strain 11-14)		

advantage to be gained from incorporating a proteolytic treatment in the assay procedure. The results are in fact lower for the papain treated extracts. The extraction procedures outlined were therefore used throughout the following work.

The effect of cyanide on the response of the assay organisms to vitamin B12

Cyanide was used in the extraction of vitamin B12-like compounds from poultry house litter and cultures of micro-organisms. Some information was therefore desirable on the effect of this reagent, in the concentration used, on the growth of the assay organisms Escherichia coli and Ochromonas malhamensis.

(a) Escherichia coli

Concentrations of vitamin B12 of 0.45, 0.15 and 0.05 µg./ml. were prepared with and without the addition of 0.01% of potassium cyanide. Using a dropping pipette, drops of each of the solutions were tested on plates seeded with Escherichia coli. After incubation it was found that no difference in zone size resulted from the addition of 0.01% of potassium cyanide.

Using a higher concentration, 0.05% of potassium cyanide, the zone size was increased at each level of vitamin B12. The zones obtained, even though larger, were still quite distinct and the zone diameter remained proportional to the logarithm

of the dose.

From this work it can be concluded that concentrations up to 0.05% of potassium cyanide can be used without interfering with the Escherichia coli plate method of assay. However the concentration of potassium cyanide in the standard vitamin B12 and unknown solutions must obviously be the same at the higher levels. The use of 0.01% potassium cyanide as has been adopted in the extraction methods is therefore satisfactory.

Similar results were obtained by Cuthbertson, Pegler, Quadling & Herbert (1951) who found that the use of 0.04% potassium cyanide in a solution containing 0.2 µg. of vitamin B12/ml. produced only a 2% error when tested against Escherichia coli by the plate method.

(b) Ochromonas malhamensis

The effect of cyanide on the growth of Ochromonas malhamensis has not been investigated but Coates & Ford (1955) have stated that the concentration of sodium cyanide in the final growth medium should not exceed 10 µg./ml. (i.e. 0.001%).

In the preparation of the standard flasks in the Ochromonas assay this concentration is not exceeded. In the assay of extracts of litter and cultures of vitamin B12-synthesizing organisms, dilution of the extract would reduce the cyanide concentration to below the inhibitory level.

The microbiological assay of vitamin B12 using Escherichia coli

Davis & Mingioli (1950) suggested that mutants of Escherichia coli which required vitamin B12 or methionine for growth might be useful for assay purposes. This suggestion was followed up by Bessell, Harrison & Lees (1950) who developed a cup-plate method for the assay of vitamin B12 using one of these mutants, a culture of which has been deposited with the National Collection of Industrial Bacteria (NCIB 8134). These workers found that the assay was subject to interference with methionine but that this was easily detected by the different appearance of the zones of exhibition. They found that a simple medium containing only ammonium dihydrogen phosphate, potassium chloride, magnesium sulphate and glucose was satisfactory for assay purposes. Further details concerning the assay procedure were given in a later paper (Harrison, Lees & Wood, 1951). Using the same test organism Burkholder (1951) developed a tube assay which showed greater sensitivity than the corresponding plate method.

Following is a description of the method of assay, using Escherichia coli, which was finally adopted in the present work for the measurement of compounds having vitamin B12 activity.

Test organism

The Escherichia coli mutant NCIB 8134 was employed.

It was maintained on nutrient agar containing 1% peptone and 1% Lab. Lemco at pH 7.0 and transferred at monthly intervals. This medium apparently supplies sufficient vitamin B12 for satisfactory growth of the organism.

Preparation of the inoculum

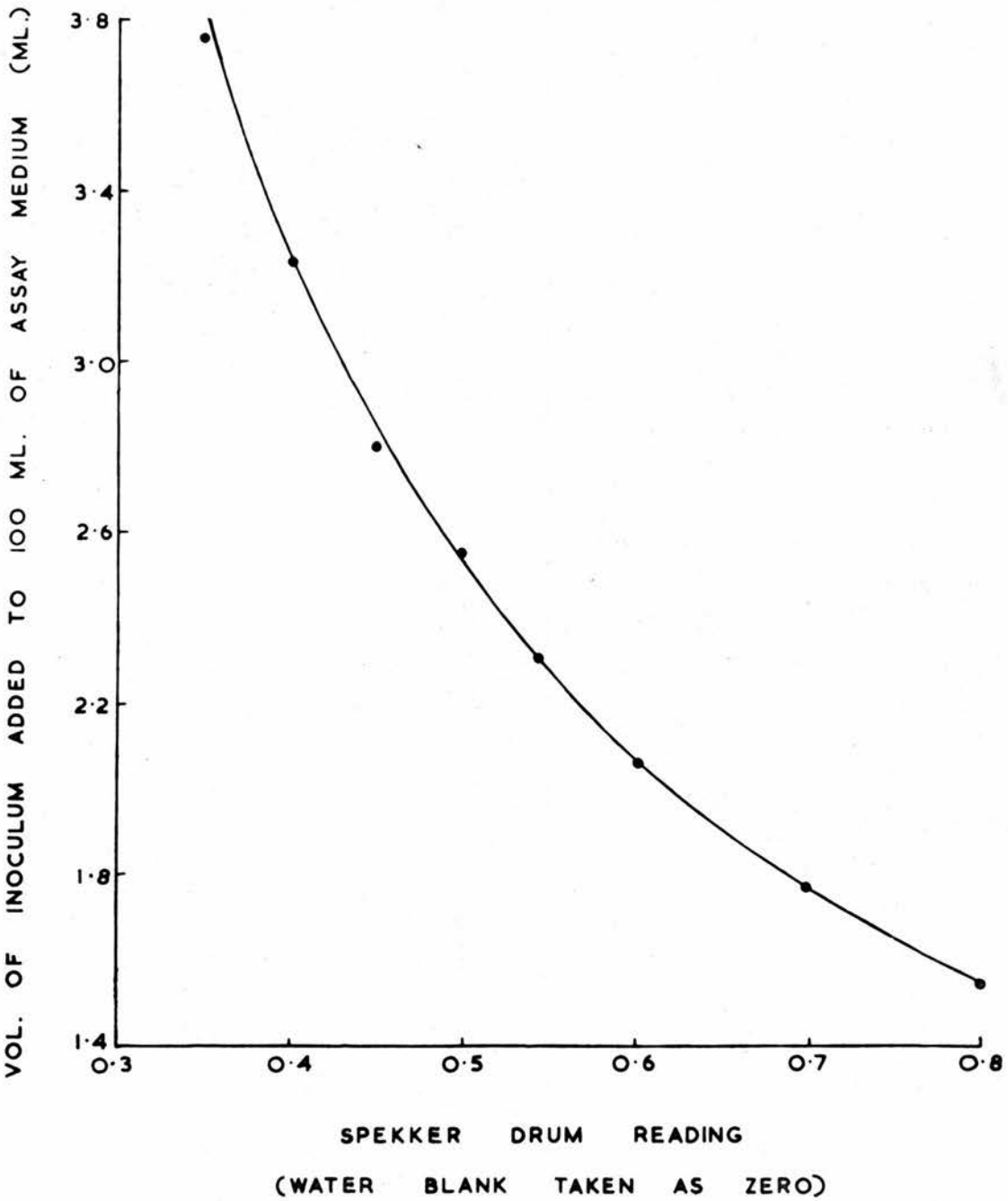
For assay purposes the organism was subcultured into nutrient broth (½% peptone, ½% Lab. Lemco, pH 7.0) and incubated at 37° for 18 hr. The culture was centrifuged for 10-15 min., the cells washed once with saline and resuspended in saline to the original volume (approx. 10 ml.). It was found that the zone size was greatly affected by the concentration of the inoculum, becoming large and diffuse and impossible to measure at low cell concentrations. To overcome this difficulty a graph was constructed relating the volume of inoculum required for the production of well defined zones, to the turbidity as measured in a Hilger Spekker photo-electric absorptiometer. This is shown in Figure 3.

Assay medium

The following medium was used for the plate assays: Glucose, 0.5 g.; $NH_4H_2PO_4$, 0.5 g.; KCl, 0.2 g.; $MgSO_4.7H_2O$, 0.05 g.; agar 1.5 g.; in 100 ml. distilled water. The pH was adjusted to 7.0 and the mixture autoclaved to dissolve the agar. After filtration and a check of pH the medium was dispensed in 100 ml. quantities in bottles and sterilized

FIGURE 3

INOCULUM REFERENCE GRAPH FOR ESCHERICHIA COLI PLATE ASSAY



at 22½ lb. momentarily.

Preparation of assay plates

The medium, melted and cooled to 45-48°, was inoculated and dispensed in 12 ml. amounts into flat-bottomed 3½ in. Petri dishes. These were allowed to set on a levelled surface. Using a background guide six plugs of agar were removed from each plate using a sterile cork borer of approximately 8.5 mm. diameter (no. 5) attached to a suction device. The plates were stored in a refrigerator if not immediately required for use. It was found that they could be kept quite satisfactorily in this way for two days but after that time the number of viable cells evidently diminished as shown by larger and more diffuse zones.

Standard vitamin B12

The standard used was Cytamen '20' (20 µg./ml. from Glaxo Laboratories Ltd.) which had previously been accurately assayed. The standard, when diluted for assay purposes, could be stored in the refrigerator for several days without loss of activity.

Preparation of sample dilutions

With the assay of members of the cobalamin series it is necessary to convert them into cyanocobalamin by treatment with cyanide. For this reason 0.5 ml. of 0.02% potassium cyanide solution was added to 0.5 ml. of the standard vitamin B12 solution and after 10 min. the mixture was further accurately diluted with phosphate buffer at pH 7.0 to give

concentrations of 0.45, 0.15 and 0.05 µg./ml. Three dilutions are advisable in order to check the linearity of the log. dose/response curve.

Similar treatment of the unknown samples was unnecessary as cyanide had already been used in the extraction procedure. Further dilution of the unknowns was made in some cases.

McIlvain's phosphate buffer at pH 7.0 was used for dilution purposes. This had the following composition per litre: 823.5 ml. 0.2M-Na_2HPO_4; 176.5 ml. 0.1M-citric acid; in distilled water. Results were consistently more linear when this buffer was used as the diluent instead of distilled water.

Filling and incubation of the plates

By means of a dropping pipette one drop of each of the three dilutions of the standard vitamin B12 was added to each of the appropriate holes on the prepared agar plates starting with the highest dilution. Similarly, using the same pipette and rinsing in sterile water between additions, samples of the unknowns were transferred to plates. Usually six or more replicates were used from each sample whether standard or unknown.

The plates were placed in the 37° incubator as soon as possible after addition of the samples (usually less than 10 min.) and incubated for approximately 18 hr. Zones were then measured to the nearest 0.5 mm. with the aid of a pair of dividers.

Calculation of results

The results for each series of replicates were averaged and for the standard vitamin B12 a graph of the logarithm of the dose against the zone diameter was plotted. For the three levels of concentration used, namely 0.45, 0.15 and 0.05 µg./ml., this should be a straight line. Using this graph the potency of any sample could be calculated knowing the zone size.

The microbiological assay of vitamin B12 using Ochromonas malhamensis

Hamilton, Hutner & Provasoli (1952) and Hutner, Provasoli & Filfus (1953) have shown that certain chrysomonads require vitamin B12 and suggested that these organisms might be useful for the assay of this vitamin. The response to vitamin B12 was linear over a wide range of concentrations and the organisms appeared to have a specificity similar to that of birds and mammals.

Ford (1953a) used one of these chrysomonads, Ochromonas malhamensis, for the assay of cyanocobalamin. Of the naturally occurring B12-vitamins only cyanocobalamin and factor I have appreciable activity for this organism; factor F has been reported as being about 5% as active as cyanocobalamin, factors G and H about 1% as active, and the remainder inactive (Brown et al., 1955; Coates & Ford, 1955). This is in marked contrast to the behaviour of Escherichia coli which responds to all these factors. As cyanocobalamin and factor I are also the only B12-vitamins which have been shown to be active for the chick (Coates & Ford, 1955) this protozoan will give a truer estimation of vitamin B12 activity for the chick.

The procedure adopted for the assay of extracts of poultry litter and cultures of micro-organisms using Ochromonas malhamensis was developed from the method used by Ford (1953a).

Test organism

The assay organism used was Ochromonas malhamensis "Pringsheim" strain, a culture of which was kindly supplied by Dr. J. E. Ford, Shinfield.

This organism failed to grow on any of the agar media commonly used in the laboratory. At first it was maintained in the basal medium at single strength (Table 31) supplemented with 0.2 mµg. of vitamin B12/ml. This was sterilized by autoclaving at 15 lb. for 15 min. and dispensed aseptically in 10 ml. amounts into sterile 50 ml. Erlenmeyer flasks. The organism was subcultured every fortnight and kept at room temperature (18-24°) at medium light intensity. Cultures grown in this way and allowed to remain at room temperature survived for up to 3 to 5 weeks both in the dark and in the light, but if kept in the refrigerator died out within a week. More satisfactory maintenance of the organism was achieved using a medium suggested by Prof. E. G. Pringsheim (personal communication). This had the following composition: Glucose, 0.2 g.; Oxoid dehydrated liver infusion, 0.1 g.; Difco Bacto peptone, 0.1 g.; in 100 ml. of water. This was sterilized by autoclaving at 15 lb. for 15 min. In this medium growth was much slower than in the synthetic medium previously used but cultures remained viable for periods of up to 6 months.

Both these media had a pH of 6.0 after autoclaving. Ochromonas malhamensis has been reported by Pringsheim (1952)

to be a distinctly, though not extremely, acidophilic organism. The optimum appears to be near pH 5.5.

Basal medium

The basal medium as devised by Ford (1953a) was used. The composition is shown in Table 31. The growth factors choline chloride, inositol, p-aminobenzoic acid, thiamine and biotin were prepared as stock solutions having the following concentrations/ml.: choline chloride, 1 mg.; inositol, 10 mg.; p-aminobenzoic acid, 1 mg.; thiamine, 1 mg.; and biotin, 10 µg. All other constituents were measured out freshly at the time of making up the medium. The five-times-strength medium was sterilized in 20 ml. quantities in 1 oz. vials at 15 lb. for 10 min. and stored in the refrigerator until required for use. 'Vitamin-free' acid hydrolysed casein (Allen & Hanbury) and Bacto 'vitamin-free' casamino acids (Difco) were both suitable forms of casein hydrolysate.

Preparation of the inoculum

For the preparation of the inoculum 10 ml. of single-strength basal medium supplemented with 0.2 mµg. of vitamin B12/ml. contained in a 50 ml. Erlenmeyer flask was inoculated with 4 drops of the stock culture of Ochromonas malhamensis. This was kept at room temperature in medium light intensity for 5-7 days. For inoculation of the assay flasks this culture was diluted 1/10 with sterile single-strength basal medium and 0.5 ml. added to each flask.

Table 31

Composition of Ochromonas medium (five-times-strength)

Casein hydrolysate (g)	5	DL-Tryptophane (g.)	0.1
Glucose (g.)	10	DL-Methionine (g.)	0.2
$(NH_4)_2H$ citrate (g.)	0.8	L-Cystine	0.1
KH_2PO_4 (g.)	0.3	Choline chloride (mg.)	2
$MgSO_4.7H_2O$ (g.)	0.2	Inositol (mg.)	10
$CaCO_3$ (g.)	0.15	p-Aminobenzoic acid (mg.)	1
'Metals' solution (ml.)	10	Thiamine (mg.)	2
$Na_2MoO_4.2H_2O$ (g.)	0.05	Biotin (µg.)	10
1% NaCN solution (ml.)	0.2	Tween 80 (ml.)	1

pH adjusted to 5.5

Distilled water to 200 ml.

The 'metals' solution had the following composition:

Ethylenediamine tetra-acetic acid (g.)	5.0
$MnSO_4.4H_2O$ (g.)	6.15
$ZnSO_4.7H_2O$ (g.)	11
$FeSO_4.7H_2O$ (g.)	1
$CoCl_2.6H_2O$ (g.)	0.25
$CuSO_4.5H_2O$ (g.)	0.04
H_3BO_3 (g.)	0.06
KI (g.)	0.001

Water to 1000 ml.

The concentration of the inoculum was not critical, the responses being not very different for dilutions of 1/5, 1/10 and 1/20. However it was important that the culture be in an active state of growth. With old cultures where the cells were losing their motility and beginning to disintegrate, very poor responses were obtained in the assay flasks.

Assay procedure

The assay was carried out in 50 ml. Erlenmeyer flasks. Before use these had been cleaned with a chromic-sulphuric acid mixture followed by thorough rinsing with water and a final rinse with distilled water. These were sterilized with cotton wool plugs and the assay then set up aseptically.

The standard vitamin B12, to which cyanide had been added as for the Escherichia coli plate assay, was diluted with sterile distilled water to give a final concentration of 0.1 mμg./ml. This was added to duplicate flasks at levels of 0.25, 0.5, 1.0, 2.0 and 4.0 ml. The volume in each flask was adjusted to 4.0 ml. with distilled water. Another pair of flasks was included containing 4.0 ml. of water with no added vitamin B12. To each flask was added 1 ml. of the five-times-strength basal medium and 0.5 ml. of the prepared inoculum. The flasks were incubated in the dark at 30° for three days. At the end of this period they were heated in a water-bath for 5 min. in order to obtain a more uniform

suspension and diluted with 5 ml. of water. Turbidities were measured in a Hilger Spekker photo-electric absorptiometer using neutral filters (H508).

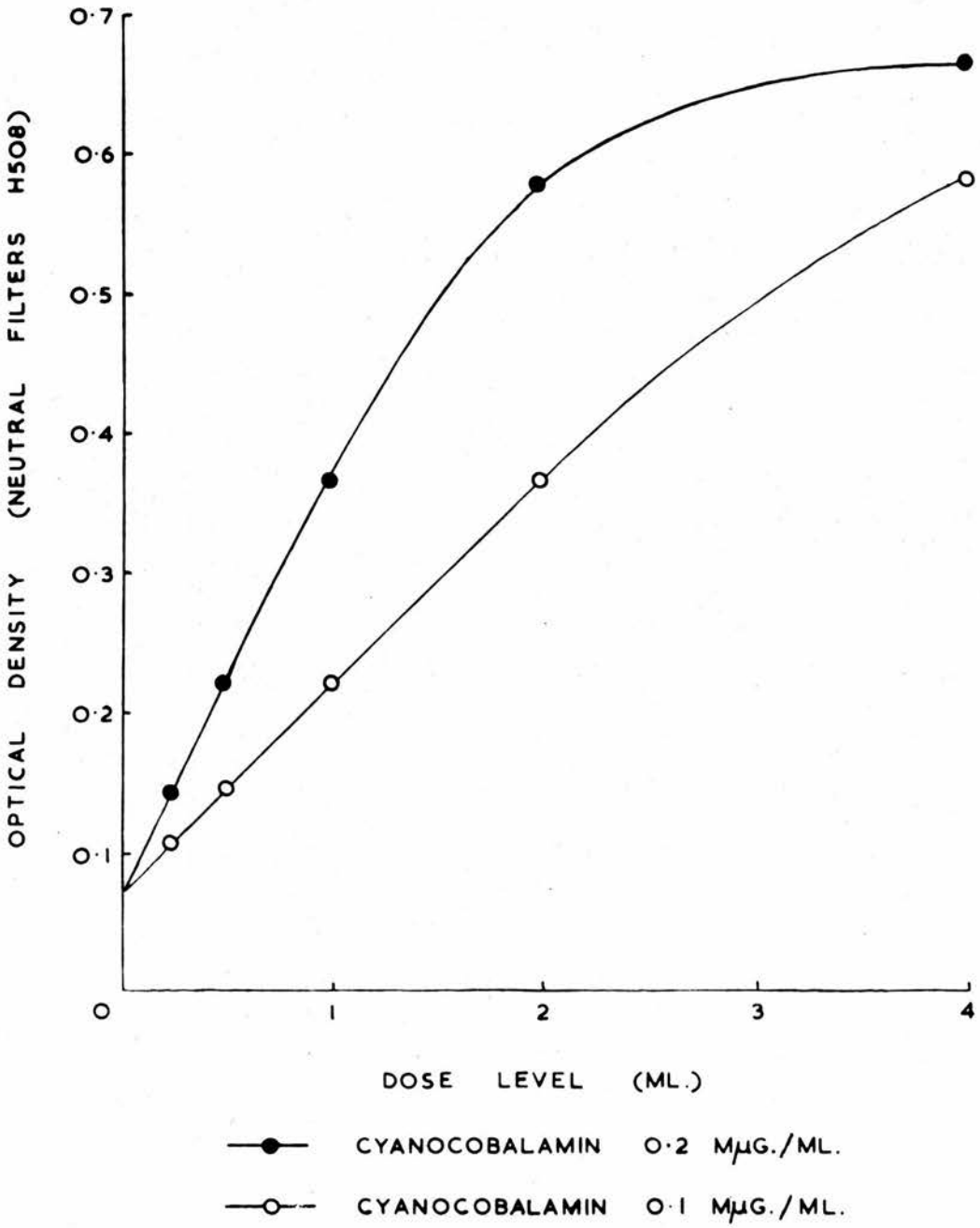
Response of Ochromonas malhamensis to vitamin B12

A concentration of 0.1 mμg./ml. was chosen for the standard vitamin B12 solution. With this standard, growth of the test organism was proportional to the concentration of vitamin B12 over almost the whole assay range as shown in Figure 4. Incubation at 30° for three days gave satisfactory growth. Lower temperatures of 27-29° gave reduced rates of growth without increasing the range of concentration for which the dose-response curve was linear.

Ford (1953a) found a linear response over a wider range of concentration than was found in this work. He however was using a shaking technique and the increased aeration probably allowed better growth of the organism particularly at the higher concentrations of vitamin B12. With a standard vitamin B12 solution containing 0.2 mμg./ml. (the concentration used by Ford) stationary cultures gave a markedly non-linear growth response as shown in Figure 4. That aeration is a limiting factor was shown by carrying out the assay in 6" x 3/4" test tubes as well as in flasks. Using a standard vitamin B12 solution of 0.1 mμg./ml. growth of the organism in the tubes was markedly less than that in the corresponding flasks at the higher vitamin B12 concentrations.

FIGURE 4

THE RESPONSE OF OCHROMONAS MALHAMENSIS TO VITAMIN B_{12}



The assay of extracts of poultry litter and cultures of micro-organisms using Ochromonas malhamensis

Extracts of poultry litter and cultures of micro-organisms were prepared as previously described. Before carrying out an accurate assay it was necessary to conduct a preliminary rough assay to determine the dilution required to give approximately 0.1 mμg. of vitamin B12/ml. To do this ten-fold dilutions up to $1/10^4$ of the extract were prepared and 1 ml. of each dilution made up to 4 ml. in an assay flask. From the growth obtained after incubation of these five flasks a rough estimate could be made of the vitamin B12 content by reference to a standard vitamin B12 curve. The extract was then suitably diluted to give approximately 0.1 mμg. of vitamin B12/ml. and an accurate assay carried out. Both the vitamin B12 standard and the test extract, in the accurate assay, should give straight line responses and intersect at the point of zero dose. From a comparison of the slopes of the lines the concentration of the unknown can be determined and multiplied by the dilution factor to give the concentration of the initial extract.

Vitamin B12 content of poultry litter

As determined by the Escherichia coli plate method

Extracts of poultry litter were assayed for their content of vitamin B12-like compounds by the Escherichia coli plate method, the results being shown in Table 32. With samples 1a and 2 no cyanide was used in the extraction and the pH was adjusted to 7.0 before the heat treatment. For all other samples the normal extraction procedure was followed. The results are also given in terms of the dry weight where the moisture contents of the samples are known.

No vitamin B12 activity was detected in the sample of unused litter (1a). For the other samples the results suggest that with increasing age the vitamin B12 activity of built-up litter increases; this is well shown with samples 4a, b, c, d, e and f, all of which originated from the same litter at different ages.

It was found, however, that this method of assay did not give valid results when applied to extracts of poultry litter. This is shown in Table 33 for several extracts where the activities, in terms of vitamin B12, were determined for the extract undiluted and the same extract diluted 1/2. The diluted extracts consistently gave higher results which means that the log. dose/response graphs of these extracts are not parallel to that of vitamin B12 itself. In a valid assay the test line must be parallel to the standard line.

Table 32

Vitamin B12 content of poultry litter
Escherichia coli plate method of assay

Sample no.	Age of litter	Vitamin B12 activity (µg./g.) Fresh weight	Dry weight
1a	Before chicks introduced	None detected	...
1b	3 months	0.50	...
1c	9 months	0.33	...
2	2 months	0.17	...
3	4 months	0.54	...
4a	1 week	0.29	0.33
4b	5 weeks	0.34	0.43
4c	9 weeks	0.31	0.45
4d	3 months	0.46	0.63
4e	4½ months	0.42	0.55
4f	9 months	0.77	1.42
5	10 weeks	0.25	0.31

Table 33

Effect of dilution on the vitamin B12 activity of extracts of poultry litter using the Escherichia coli plate method of assay

Sample no.	Vitamin B12 activity (mμg./ml. of extract) Extract undiluted	Extract diluted 1/2
1c	33.2	42.8
3	54.1	76.6
+ 4a	22.0	30.6
4b	34.0	44.0
4c	31.2	47.0
4d	45.9	58.0
4e	41.9	52.6

+ 1/13 suspension

Other workers have also reported non-parallelism when using the Escherichia coli plate method of assay. Thus Ford (1952) found factor B to give a non-parallel response in terms of vitamin B12 and the same author (Ford, 1953b) reported that the response to pseudovitamin B12 by E. coli was markedly non-linear. Similar non-linear responses have also been reported for factor F (the main factor in chicken faeces) and factor I by Brown et al. (1955) using the E. coli plate assay. When estimating the vitamin B12 activity of the organs and excreta of sheep by the E. coli method Dawbarn & Hine (1954) found that the results were non-parallel in many cases.

As the diluted extracts give larger zones than would be expected it was thought that the undiluted extracts might be interfering with the diffusion of the vitamin in the Escherichia coli seeded plates. In order to investigate this further it was decided to measure the zone size obtained by adding vitamin B12 to an extract of litter in which vitamin B12 activity had been destroyed and compare this with the zone size obtained from the same concentration of vitamin B12 in phosphate buffer.

Heating extracts in an alkaline medium has been used by various workers for the destruction of vitamin B12. Almost the whole of the vitamin B12 activity in extracts of gut contents and faeces is destroyed by autoclaving at 15 lb.

for 15 min. at pH 11.6 (Coates et. al., 1953) and vitamin B12 activity is effectively destroyed in extracts of milk by the same treatment (Gregory, 1954). Similarly Hoffmann, Stokstad, Hutchings, Dornbush & Jukes (1949) destroyed 97% of the vitamin B12 activity in liver extract by heating with 0.2N-NaOH at 100° for 30 min.

A 1/10 suspension of a typical built-up litter sample was prepared in the usual way with addition of 0.01% of potassium cyanide. This was divided into two 300 ml. quantities one of which was adjusted to pH 5.0 with N-H_2SO_4, the other to pH 11.5 with N-NaOH. Both were autoclaved at 15 lb. for 15 min. The pH of the acid extract, after autoclaving, was 6.0 so the alkaline extract was adjusted to the same pH by addition of N-H_2SO_4. The two extracts were clarified by centrifuging and resterilized by Seitz filtration. One drop of each of the extracts was tested on an Escherichia coli seeded plate. This showed that the activity had only been partially destroyed by the alkali treatment. To gain more quantitative information the two extracts were assayed for their vitamin B12 content using Ochromonas malhamensis. This gave the following results:

Normal extraction at pH 5.0	58.7 mμg./ml.
Extraction at pH 11.5	27.0 mμg./ml.

From these results it can be seen that autoclaving at 15 lb. for 15 min. at pH 11.5 is not effective in destroying the vitamin B12 present, in fact only 54% has been inactivated.

Because of the lack of specificity displayed by this mutant of Escherichia coli it was not thought worth while to pursue this problem. This assay organism responds to all the naturally occurring vitamin B12-like factors so that the results, even if valid, would not be a measure of the vitamin B12 activity of these extracts for the chick. The results obtained using Ochromonas malhamensis would give a better idea of the true potency. The assays were therefore repeated using this organism.

As determined with Ochromonas malhamensis

Assays of the extracts were carried out as previously described for Ochromonas malhamensis. The results are shown in Table 34. Samples 4a, b, c, d, e and f were from the same litter at different ages and the figures show that an increase in the vitamin B12 content does occur with the age of the litter. Sample 7 gave a higher result than the others but this litter contained cow manure which probably contributed to its vitamin B12 content.

The values for vitamin B12 activity using Ochromonas malhamensis are lower than those obtained employing Escherichia coli as the assay organism; this is attributed

Table 34

Vitamin B12 content of poultry litter
Microbiological assay with Ochromonas malhamensis

Sample no.	Age of litter	Vitamin B12 activity (µg./g.) Fresh weight	Dry weight
1c	9 months	0.17	...
3	4 months	0.50	...
4a	1 week	0.09	0.10
4b	5 weeks	0.17	0.21
4c	9 weeks	0.19	0.28
4d	3 months	0.35	0.47
4e	4½ months	0.34	0.45
4f	9 months	0.29	0.53
5	10 weeks	0.14	0.17
6	7 months	0.27	0.31
7	9 months	0.85	1.46

to the response of the latter assay organism to compounds other than cyanocobalamin.

The Ochromonas results are higher than those reported by Halbrook, Sutton & Winter (1950) for built-up litter samples. These workers used Lactobacillus leichmannii (ATCC 4797) as the test organism and found that unused corn cob litter gave an average value of 1.1 mμg. of vitamin B12/g., the value rising to 50 mμg./g. at 5 weeks and 261 mμg./g. at 1 year. These lower results could be due to the fact that these workers apparently did not use cyanide in the extraction of their samples.

It is interesting to note that Ford (1953a), using Ochromonas malhamensis as the assay organism, found a value of 0.35 μg./g. for the vitamin B12 activity of fish solubles. This is comparable with the values found for built-up litter samples.

As Ochromonas malhamensis responds almost specifically to cyanocobalamin it would appear, from these results, that built-up poultry litter could act as a source of this vitamin for the birds.

Vitamin B12 production by micro-organisms isolated from poultry litter and droppings

The isolation of vitamin B12 from liver stimulated interest in the possibility of its synthesis by micro-organisms. Large numbers of isolates were subsequently tested by various workers and it was found that a great variety of the eubacteriales and actinomycetales did possess this ability. Most of the isolates have originated from soil and very little attention has been paid to built-up poultry litter as a source even though the latter has been shown to possess "animal protein factor" activity.

During the present investigation a large number of micro-organisms were isolated from poultry litter and droppings. These were screened for those capable of producing significant amounts of vitamin B12-active compounds. Some factors influencing the synthesis of vitamin B12 by representative cultures were also investigated.

Screening of isolates

The cultures to be tested for vitamin B12 production were subcultured on to nutrient or peptone-yeast extract agar slopes and incubated at 22°. From the resulting growth a light suspension of each organism was made in saline of which 0.1 ml. was transferred to 5 ml. of a vitamin B12-deficient medium. This medium was the same as

that used by Burton & Lochhead (1951) and contained per litre: K_2HPO_4, 1.0g.; KNO_3, 0.5 g.; $MgSO_4.7H_2O$, 0.2 g.; $CaCO_3$, 0.1 g.; NaCl, 0.1 g.; $FeCl_3.6H_2O$, 0.01 g.; $CoCl_2.6H_2O$, 0.008 g.; in distilled water. After the salt mixture was heated and filtered the following were added: Difco casamino acids (vitamin-free), 5.0 g.; glucose, 10.0 g.; and Difco yeast extract, 1.0 g. The medium was adjusted to pH 7.2 and sterilized by autoclaving at 15 lb. for 15 min. The inoculated tubes were incubated at 22° for 14 days.

In order to test for the presence of vitamin B12-active substances the cultures were extracted as previously described on p. 99 . One drop of each extract was then tested, in duplicate, on a plate seeded with Escherichia coli. After overnight incubation at 37° cultures showing zones of exhibition were noted.

None of the 25 moulds or 22 yeasts tested showed any vitamin B12 activity. Thirty-three cultures of enterococci also failed to produce any activity. Of 769 other isolates tested, 89 showed vitamin B12 activity by this method, that is 11.6%. Although the response of Escherichia coli is not specific for cyanocobalamin, representatives of these 89 isolates were found to be also capable of supporting the growth of Ochromonas malhamensis in a vitamin B12-deficient medium.

Hall, Benjamin, Bricker, Gill, Haynes & Tsuchiya (1950)

similarly found moulds and yeasts to produce no vitamin B12-like compounds. In contrast Halbrook, Cords, Winter & Sutton (1950) have listed moulds and yeasts among their vitamin B12-producing organisms. These latter workers did not check, as did Hall and his associates, that the substances stimulating growth of the test organism, Lactobacillus leichmannii, were similar to vitamin B12. Thus their statement that moulds and yeasts can synthesize vitamin B12 is questionable.

Halbrook, Cords, Winter & Sutton (1950) reported also that only 2.8% of 142 isolates from poultry house litter and droppings produced no detectable quantity of vitamin B12 (< 0.01 mμg./ml. of broth culture), 32.3% produced between 0.01 and 0.10 mμg./ml., 22.5% produced between 0.10 and 0.4, and 42.2% more than 0.4 under the conditions employed. Here again there was no check on the nature of the substances stimulating growth of the test organism and it is not known just how much reliance should be placed on these figures.

Burton & Lochhead (1951), in studying the production of vitamin B12-active substances by micro-organisms, also tested isolates from poultry house litter using the Lactobacillus lactis Dorner (ATCC 10,697) agar plate method. Of the isolates tested, 55.6% showed detectable amounts of vitamin B12 by this method. This is a higher percentage than was found in the present investigation using

Escherichia coli as the screening organism. This may be due either to a difference in sensitivity between the two test organisms used, or to non-specific responses by *L. lactis*.

Types of micro-organisms producing significant amounts of vitamin B12

The 89 positive isolates belong to the following genera and are discussed in more detail in Part III:

Corynebacterium, 28 strains

Nocardia, 1 strain

Streptomyces, 3 strains

Pseudomonas, 26 strains

Alcaligenes, 10 strains

Achromobacter, 16 strains

Flavobacterium, 5 strains.

As the isolates described originated from a variety of media, some of which were selective, the relative numbers of the various types do not represent the proportions in which they exist in poultry litter. However, corynebacteria are obviously the most important types numerically since they all originated from high dilution plates used for estimating the total count.

No reference has been found in the literature to vitamin B12 production by corynebacteria but Burton & Lochhead (1951) have reported strains of *Nocardia* which have this property. Strains of *Streptomyces* have been

found by many workers to be active producers of vitamin B12 (Saunders, Otto & Sylvester, 1952).

Vitamin B12 production is a common feature of the pseudomonads isolated during this work. Burton & Lochhead (1951) also mentioned pseudomonads among their positive isolates.

Four of the flavobacteria were identified as Flavobacterium solare. This is of interest since Petty & Matrishin (1949) isolated an organism, of probable chicken faeces origin, which they tentatively identified as F. solare. A concentrated culture of this organism possessed APF activity and was capable of stimulating chick growth.

Factors influencing vitamin B12 production under laboratory conditions

An understanding of the factors which influence vitamin B12 production by micro-organisms under laboratory conditions would give some indication of the conditions in built-up litter which would be most favourable for microbiological synthesis of the vitamin. With this idea in view, representative strains of the vitamin B12-producing organisms were selected for further study.

From the literature pertaining to the production of vitamin B12 by micro-organisms it would appear that, for any one strain, synthesis of the vitamin is roughly proportional to the growth of the organism and maximum synthesis occurs under optimal conditions for growth.

Seven isolates were selected for further study. These included four corynebacteria representative of the B12-producing types, one nocardia, one pseudomonad and one flavobacterium strain.

From the growth on a peptone-yeast extract agar slope, a light suspension of each organism was made in saline and 0.1 ml. inoculated into 10 ml. of the medium being investigated. After incubation for 14 days, the volume of medium was re-adjusted to 10 ml. with distilled water.

The turbidity of each culture was measured in a Hilger Spekker photo-electric absorptiometer using neutral filters (H508), uninoculated medium being taken as having a zero reading. As the drum readings, on the instrument used, are not quite proportional to the amount of growth, a correction was made to the turbidity readings so that they did represent the relative degree of growth for any one organism. If a culture gave a drum reading of >1 it was diluted 1/2 or 1/4 so that the drum reading became <1; the turbidity reading (corrected) was then multiplied by the dilution factor.

The cultures were also assayed for their vitamin B12 content after extracting by the method previously outlined. Ochromonas malhamensis was employed as the assay organism.

All glassware used in the experiments was cleaned in a sulphuric-chromic acid solution and thoroughly rinsed with water before use.

(a) The effect of medium and temperature of incubation

In this experiment the effects of the composition of the medium and of the temperature of incubation on the growth and vitamin B12 production of the test organisms were examined.

Both a simple and a complex medium were used. The complex medium II was the vitamin B12-deficient medium used for the screening of the isolates; this contained inorganic salts, vitamin-free hydrolysed casein, glucose and yeast extract. The simple medium I contained the same mixture of inorganic salts and glucose with the addition of 0.5% $NH_4H_2PO_4$. Both media were adjusted to pH 7.0 and sterilized at 15 lb. for 15 min. Ten ml. quantities of each medium were dispensed aseptically into 6" x 3/4" test tubes and after inoculation incubated at 22°, 30° and 37°.

Only the two Gram-negative organisms were capable of growth in the simple medium containing ammoniacal nitrogen and only the flavobacterium showed growth at 37°. The turbidity measurements and corresponding values for vitamin B12 in mμg./ml. of culture are shown in Table 35. Also included for each culture, in brackets, is the ratio

$$\frac{\text{vitamin B12 concn.}}{\text{turbidity}}$$

which provides a measure of the efficiency with which vitamin B12 is produced by any one organism.

From the results it can be seen that for cultures grown in medium II, the concentration of vitamin B12 decreases

Table 35

The effect of medium and temperature of incubation on the growth and vitamin B12 production by micro-organisms

		Med. I 22°	Med. II 22°	Med. I 30°	Med. II 30°	Med. I 37°	Med. II 37°
Corynebacterium 12-3	Turb. B12	...	0.73 6.4 (8.8)	...	0.87 5.5 (6.3)	...	...
Corynebacterium 8-29	Turb. B12	...	0.83 5.8 (7.0)	...	1.34 5.8 (4.3)	...	...
Corynebacterium 11-5	Turb. B12	...	0.33 9.6 (29.9)	...	0.40 4.2 (10.5)	...	...
Corynebacterium 11-12	Turb. B12	...	1.04 18.9 (18.2)	...	0.47 7.8 (16.6)	...	...
Nocardia 13-26	Turb. B12	...	0.73 7.1 (9.7)	...	2.20 6.5 (3.0)	...	...

Table contd. on next page

Table 35 (contd.)

Pseudomonas 8-G11	Turb	0.20	1.24	0.52	1.46		
	B12	1.4	10.4	2.2	4.5	...	...
		(7.0)	(8.4)	(4.2)	(3.1)		
Flavobacterium 8-21	Turb.	0.45	0.79	0.40	0.90	0.30	0.36
	B12	1.6	7.8	1.2	4.6	0.58	0.82
		(3.6)	(9.9)	(3.0)	(5.1)	(1.9)	(2.3)

The concn. of vitamin B12 is given in mμg./ml. of culture.
The figure in brackets, for each culture, is the ratio B12 concn./turbidity.
Med. I is a simple medium containing ammoniacal nitrogen; med. II is nutritionally a more complex medium.

with increasing temperature of incubation except for strain 8-29 where the figure is the same at both 22° and 30°. For cultures in medium I, although strain 8-G11 shows a greater concentration of vitamin B12 at 30° than at 22°, strain 8-21 shows a decrease in B12 concentration with increasing temperature. An examination of the figures for the efficiency of vitamin B12 production reveals reduced efficiency as the temperature of incubation rises. This applies to both media.

In general, therefore, the concentration of vitamin B12 and the efficiency with which it is produced both decrease with increasing temperature even though cell yields may be higher at the more elevated temperatures.

(b) The effect of aeration

In this experiment a comparison was made between cultures grown in 6" x 3/4" test tubes and in 100 ml. Erlenmeyer flasks in which the medium formed a shallow well-aerated layer. Ten ml. quantities of medium II were used; cultures were incubated at 22°. As before, turbidities and vitamin B12 concentrations in mμg./ml. of culture were determined, the results being given in Table 36.

For the pseudomonad, growth was practically as good in the tube as in the flask possibly due to its motility. This was the only strain which showed less vitamin B12 production under the more aerated conditions; efficiency of production

Table 36

The effect of aeration on growth and vitamin B12 production by micro-organisms

		Test tube	Flask
Corynebacterium 12-3	Turb.	0.35	1.98
	B12	2.9	20.6
		(8.3)	(10.4)
Corynebacterium 8-29	Turb.	0.48	1.74
	B12	5.1	22.0
		(10.6)	(12.7)
Corynebacterium 11-5	Turb.	0.52	1.40
	B12	14.2	33.6
		(27.3)	(24.0)
Corynebacterium 11-12	Turb.	0.70	2.80
	B12	18.2	68.0
		(26.0)	(24.3)
Nocardia 13-26	Turb.	0.73	2.22
	B12	7.1	18.2
		(9.7)	(8.2)
Pseudomonas 8-G11	Turb.	0.94	1.26
	B12	6.2	4.7
		(6.6)	(3.7)
Flavobacterium 8-21	Turb.	0.40	1.92
	B12	5.0	15.6
		(12.5)	(8.1)

The concn. of vitamin B12 is given in mμg./ml. of culture. The figure in brackets, for each culture, is the ratio B12 concn./turbidity.

was also less. For the other six strains growth was markedly improved with greater aeration; the concentration of vitamin B12 was correspondingly increased, the efficiency of production remaining approximately the same.

Discussion of results. From these results it would appear that the growth and B12 production by these organisms is most affected by the aeration of the medium. This particularly applies to the corynebacteria which have previously been shown to be the most numerous of the B12-synthesizing organisms in built-up poultry litter; abundant aeration results in markedly improved growth with a corresponding increase in the production of vitamin B12. It therefore seems likely that the production of vitamin B12 in built-up poultry litter will be favoured when it is kept in a moderately dry and friable, and hence well-aerated, condition.

For any one organism it was found that the production of vitamin B12 became less as the maximum temperature for growth was approached. In practice, however, the temperature in built-up poultry litter has never been found to become sufficiently high for it to have any adverse effect on the production of vitamin B12 within the material. Very low temperatures would, of course, be expected to depress vitamin B12 production by restricting growth of the organisms concerned.

Action of micro-organisms on vitamin B12

Many isolates from poultry litter and droppings were found which produced no detectable vitamin B12 activity when tested on plates seeded with Escherichia coli. The possibility existed that these strains might decompose vitamin B12. Representative strains of the dominant types were therefore selected and their action on vitamin B12 investigated. The strains tested included 23 corynebacteria, 1 nocardia, 7 micrococci, 1 pseudomonad, 2 alcaligenes, 2 achromobacter and 1 cytophaga as well as Bacterium globiforme, Bacterium linens, Corynebacterium ureafaciens, Bacterium ammoniagenes and Topping's cultures 81 and 178.

From the growth on a peptone-yeast extract agar slope, a light suspension of each organism was made in saline and 0.05 ml. inoculated into 5 ml. of peptone-yeast extract broth to which had been added 0.08 µg. of vitamin B12/ml. Cultures were incubated at 22° for 14 days and then extracted by the method adopted for cultures of micro-organisms. One drop of each extract was tested, in triplicate, on plates seeded with Escherichia coli. An uninoculated tube of medium was extracted and tested in the same way. After overnight incubation at 37° the zones of growth were measured and each series of replicates averaged.

It was found that in no case had the amount of vitamin B12 in the medium decreased, the zones being all equal in size

to that of the uninoculated control tube.

This does not exclude the possibility of the existence in poultry litter of organisms which are capable of decomposing vitamin B12. They are not, however, among the dominant types. If destruction of vitamin B12 does occur in built-up litter it must be more than compensated for by fresh synthesis, because under normal conditions the vitamin B12 content increases with the age of the litter.

SUMMARY

A satisfactory method for the measurement of vitamin B12 in poultry litter and cultures of micro-organisms, using *Ochromonas malhamensis* as the test organism, has been developed.

For built-up litter samples potencies up to 0.53 µg. of vitamin B12/g. dry weight were found. The concentration was shown to increase with the age of the litter. One sample containing cow manure had a potency of 1.46 µg. of vitamin B12/g. dry weight.

The use of a mutant of *Escherichia coli*, although not satisfactory for assays on account of its lack of specificity, proved useful for the screening of isolates for those capable of forming significant amounts of vitamin B12-active substances.

Corynebacteria are the most important vitamin B12 synthesizers. Other organisms which were found to produce the vitamin belong to the genera *Nocardia*, *Streptomyces*, *Pseudomonas*, *Alcaligenes*, *Achromobacter* and *Flavobacterium*.

Factors influencing vitamin B12 production under laboratory conditions were investigated for representative strains. For any one organism vitamin B12 production was greater in a complex than in a simple medium incubated under the same conditions; generally the vitamin B12 yield and the efficiency of production became less as the temperature of incubation was raised even though the cell yield was frequently greater. Increased

aeration improved growth and vitamin B12 production by all the non-motile strains tested but one pseudomonad showed slightly less vitamin B12 under the more aerated conditions. In relating these findings to built-up litter it would be reasonable to assume that production of vitamin B12 would be most favoured when the litter material is of a friable consistency and hence freely aerated, also when it is not excessively cold which would otherwise retard growth of the organisms concerned.

No organisms capable of decomposing vitamin B12 were found among the dominant flora of poultry litter and droppings.

SUMMARY

SUMMARY

The total viable count of built-up poultry litter was found to lie in the region of 10^{10} to 10^{11}/g. fresh weight and appeared to be little affected by factors such as age, temperature, moisture content and pH. Counts for unused litter and poultry droppings were of a lower order of magnitude. In built-up litter of high alkalinity coryne-bacteria were the predominant bacterial types; micrococci occurred sporadically and small numbers of nocardia, strep-tomyces, aerobic spore-formers, streptococci and a variety of Gram-negative bacteria were encountered. The number of Gram-negative organisms appeared to be influenced by the alkalinity of the sample; they were less abundant in litters where the pH and buffering capacity were high. Strongly alkaline conditions also tended to lower the numbers of fungi but had no effect on the count of enterococci.

It was found that the decomposition of uric acid in built-up poultry litter is brought about almost exclusively by the action of aerobic bacteria. Organisms attacking uric acid usually comprised about one-third of the total flora present and included strains of Corynebacterium, Nocardia, Streptomyces, Alcaligenes and Achromobacter. Ammonia is sometimes formed as a result of uric acid breakdown but with most strains decomposition proceeds only as far as urea. This is in turn hydrolysed, with the release of ammonia, by other

strains identified as belonging to the genera Corynebacterium, Micrococcus, Alcaligenes, Achromobacter and Cytophaga. It is suggested that the ammoniacal smell and high alkalinity of built-up poultry litter result largely from the microbiological decomposition of uric acid.

For the estimation of vitamin B12 in extracts of poultry litter and cultures of micro-organisms an assay method using Ochromonas malhamensis, an organism specific for vitamin B12, was used. For built-up litter potencies up to 0.53 µg. of vitamin B12/g. dry weight were found; one sample containing cow manure had a potency of 1.46 µg. of vitamin B12/g. dry weight. A mutant of Escherichia coli proved useful for the screening of isolates; about 10% of all the cultures tested produced detectable amounts of vitamin B12-active compounds. These belonged to the genera Corynebacterium, Nocardia, Streptomyces, Pseudomonas, Alcaligenes, Achromobacter and Flavobacterium. Some of the factors influencing vitamin B12 production under laboratory conditions were investigated for representative strains. Of these the most important for high B12 yields was abundant aeration. It would thus be reasonable to assume that, in litter of a friable consistency, the synthesis of vitamin B12 is favoured.

Corynebacteria were the most numerous types encountered during this study of poultry litter. Many of these appear to be closely related to the Bacterium globiforme group

normally present in soil. The characteristics of these and of other isolated strains are given and their possible identity discussed. It is considered that they should be recognized as members of the genus Corynebacterium and not of Arthrobacter. Organisms belonging to other genera have been described and where possible identified with named species.

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